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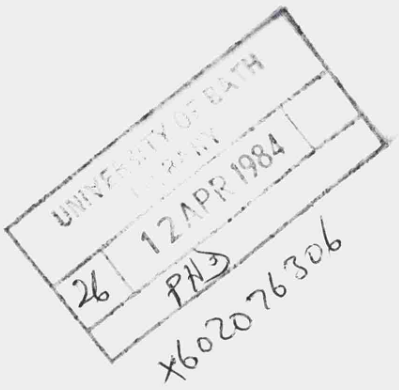
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THE INTERACTION OF THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE* (Sorokin) AND THE INSECTICIDE DIFLUBENZURON ON *MANDUCA SEXTA* (JOHANNSON)

Submitted by A.E.M. Hassan

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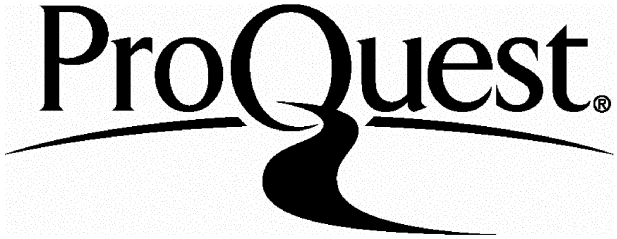

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ABSTRACT

A study was made of the susceptibility of young larvae of the tobacco hornworm moth, *Manduca sexta*, to the action of the benzoylurea diflubenzuron (Dimilin). This insecticide was a very effective stomach poison but ED50 values were lower when insects were maintained in an atmosphere with high rather than low relative humidity. The susceptibility of the insect also depended on the stage (instar) of the insect and its diet. In contrast to its effects on many other insect species, Dimilin exhibited contact toxicity against *Manduca* and high stomach doses reduced faecal output.

There being no previous reports of the isolation of a parasitic fungus from *Manduca*, a screen was performed using isolates of four species of entomopathogenic fungus, *Metarhizium anisopliae* (M.E.I.) proved to be very effective and a reproducible bioassay was developed using a dipping technique. Interference from antifungal compounds in the artificial diet was circumvented by feeding experimental insects on tomato leaves. Conidia which were presoaked (P) for 20 h in distilled water prior to inoculation on *Manduca* larvae germinated more quickly than fresh conidia (F); as a consequence pre-soaked conidia were more pathogenic than fresh.

Dual applications of Dimilin (ED50 dose) and *Metarhizium anisopliae* (ED50 dose) were synergistic in their action against

second instar *Manduca* larvae (using Benz's (1971) criteria). The greater efficiency of pre-soaked conidia (P) was still apparent when they were applied in combination with Dimilin (D). DP treatment killed significantly more second instar larvae than DF. It was hoped that the greater pathogenicity of P and DP treatments would be translated into a reduced period of high relative humidity necessary for the initiation of infection. However, the results were disappointing.

Most deaths in the combined insecticide/fungus treatments were due to mycosis, adding support for the hypothesis that the action of Dimilin on the cuticle facilitated the entry of the fungus into the insect. An ultrastructural study was carried out to seek direct support for this conclusion. Treatment with Dimilin did not stop the growth of abdominal tergal cuticle. Post-ecdysial Dimilin-affected cuticle, however, was characterised by the absence of lamellae and presence of globules of melanin-like material. Lateral growth of *Metarhizium* between the lamellae of normal cuticle was a prelude to vertical penetration primarily via pore canals. Progress towards the epidermis apparently did not occur in a step-wise fashion.

Cuticle of Dimilin-treated insects provided little resistance to penetration by hyphae of *Metarhizium*. Widespread histolysis of post-ecdysial cuticle occurred. In addition, although lamellate pre-ecdysial cuticle was not affected by Dimilin, the cuticle of the pore canals was similar in

appearance to, and presumably laid down at the same time as, the post-ecdysial cuticle. Pore canals were, therefore, areas of weakness in the pre-ecdysial cuticle which as a consequence failed to provide a mechanical barrier to the penetrating fungus.

CONTENTS

GENERAL INTRODUCTION

Synthetic insecticides remain the most effective means of controlling insect populations. However, the detrimental effects of chemical insecticides on the environment and the problems of resistance and pest resurgence have led to a search for alternative methods of pest control. So called "biological control" has taken the form of mass release of parasites and predators and the introduction of sterile males. In addition, success has been achieved by the dissemination of bacterial, viral and protozoan diseases. Although the exploitation of entomopathogenic fungi for pest control has been hampered by a lack of knowledge, there is renewed interest and there are a number of notable successes (see Introduction to Chapter 2).

The entomopathogenic fungi do not occupy a definite systematic place, but are distributed among the four major groups, viz. *Deuteromycetes, Phycomycetes, Ascomycetes* and *Basidiomycetes*. The most commonly encountered entomopathogens of the *Deuteromycotina* belong to only a few genera, namely *Beauveria, Metarhizium* and to a lesser extent *Aspergillus, Hirsutella* and *Nomuraea* (Madelin, 1966), all are facultative parasites. In contrast, the *Phycomycotina* e.g. *Entomophthora* and *Coelomomyces* and the *Ascomycotina* e.g. *Cordyceps,* are mostly obligate necrotrophs. There are only a few representatives in the *Basidiomycetes* e.g. the genus *Septobasidium* which are parasitic on colonies of scale insects (Ferron, 1978).

The general biology of entomopathogenic fungi has been reviewed many times (e.g. Madelin, 1963; Müller-Kögler, 1965; Roberts and Yendol, 1971; Ferron, 1978; Roberts and Humber, 1981). The cycle of infection by most conidial entomopathogenic fungi starts with the attachment of the conidium to the insect cuticle. The importance of this event is clear from the fact that very few conidia of a hypovirulent mutant of *Metarhizium anisopliae* attached to the perispiracular valves of the mosquito *Culex pipiens*, compared with those of the virulent wild type (Al-Aidroos and Roberts, 1978). Cuticular penetration is initiated either directly by a germ tube or by the production of an appressorium which attaches firmly to the cuticle and sends out an infection peg (Mohamed *et al.*, 1978; Robinson, 1966 and McCauley *et al.*, 1968). Penetration of the cuticle is generally considered to involve enzymic and mechanical components (Ferron, 1978; Mohamed *et al.*, 1978). The arthrodial membrane at joints and between segments is often the favoured site of penetration by entomopathogenic fungi (David, 1967; Schabel, 1978). However there are reports of invasion via sense organs (McCauley *et al.*, 1968), segmental cuticle (Mohamed *et al.*, 1978),and the alimentary canal (David, 1967; Ferron, 1978).

On entering the haemolymph penetrant hyphae of certain entomopathogenic fungi continue filamentous development as with *Entomophthora coronata* in the termite, *Reticulitermes flavipes* (Yendol and Paschke, 1965) and *Aspergillus flavus* in the silkworm, *Hyalophora cecropia* (Sussman, 1952). However,

most, sooner or later, produce hyphal bodies which circulate in the haemolymph before germinating into mycelium (Prasertphon and Tanada, 1968).

There may be limited (Brobyn and Wilding, 1977) to extensive (Mohamed *et al.*, 1978) degradation of host tissue prior to death, but often fungal growth is confined to the haemolymph during the parasitic phase. However, *Entomophthora coronata* and *E. apiculata* are found in the fatbody, cuticle and other tissues of *Galleria mellonella*, probably because the hyphal bodies are too large to circulate in the haemolymph (Prasertphon and Tanada, 1968).

It is unlikely that death from mycosis is the result of a single lethal lesion. Indeed it is difficult to define the point of death of an insect since heart and brain are not essential to life and lungs are absent. Host physiology must be chronically disrupted by the growth of hyphae through tissues and blockage of the haemocoel by hyphal bodies (Madelin, 1963), resulting in stress reactions including autointoxication (Sternberg, 1963). In those cases where fungi overcome their hosts' after limited growth, toxins may play a significant part in host death, perhaps particularly by causing neuro-muscular dysfunction (Bell, 1974; Roberts, 1980). Toxins may elicit further pathological effects by causing water loss from cells generally and Malpighian tubules in particular (Zacharuk, 1973). Roberts (1980) has also suggested that, in general, the lower entomopathogenic fungi (e.g. *Coelomomyces*,

Lagenidium and *Entomophthora*) overcome susceptible hosts primarily by "starvation", that is they use up soluble host reserves, rather than via toxins. Following the death of the host the mycelium spreads rapidly to fill the whole body. The fungus can remain dormant within the mumified insect for long periods, particularly when conditions are dry. Given favourable temperature and high humidity hyphae emerge through the integument and sporulation occurs. The latter can take place immediately after the death of the host without the intervention of a dormant phase (Madelin, 1963).

The invasion of the insect cannot be considered successful until the fungus reaches the epidermis, because the inoculum may be lost with the exuvia if ecdysis intervenes (Zacharuk, 1973; Vey and Fargues, 1977). Consequently a premium must be placed on speed of germination and penetration particularly in those insects with short intermoult periods. Thus *Nomuraea rileyi* invades *Anticarsia gemmatalis* within 6 h of application (Boucias and Pendland, 1982) while *Verticillium lecanii* may cause death of aphids 24 h post-inoculation (Hall, 1980). In the light of the above it is not surprising that hyper-virulence in certain mutant of *M. anisopliae* pathogenic for *Culex pipiens* correlated with speed of germination (Al-Aidroos and Roberts, 1978; Al-Aidroos and Seifert, 1980).

A further constraint on successful invasion is the need for a high atmospheric relative humidity during spore germination. Any means of reducing germination time is therefore likely to increase the efficacy of entomopathogenic fungi for biological control (see Chapter 3).

The insect cuticle provides the first of a series of barriers to infection. Some comparison of the pathogenicity of topically applied and ingested spores suggest that cuticle *per se*, may play a part in strain specificity. Ferron and Diomandé (1969) found that the Scarabaeid beetle, *Orycetes monoceros*, was susceptible to topically applied spores only from strains of *M. anisopliae* isolated from *Orycetes* spp. Additional isolates were only pathogenic by injection. It remains to be seen whether the cuticle provides a specific chemical barrier or is purely a general physical barrier which optimises the efficiency of the haemocytic defences. In relation to this fungitoxic lipids have been extracted from the cuticle of a numberof insects (Koidsumi, 1957; Evlakhova and Shekhurina, 1963) and lipids extracted from strains of *Bombyx mori* resistant to *Aspergillus flavus* had greater antifungal activity than those from susceptible strains (Koidsumi and Wada, 1955). The inhibitory chemical in the cuticle of *Spodoptera* ~~*frugiterda*~~ fugiperda has been identified as caprylic acid (Smith and Grula, 1982). However, until the *in vivo* concentration of these substances has been determined, their significance cannot be certain.

In addition to the passive defences against entomopathogenic fungi described above, the host responds actively to invasion. Dark patches in the cuticle surrounding a penetrant hypha are characteristic of early symptoms of infection (Wallengren and Johnson, 1929; Nirula, Radha and Menon, 1955; Takahashi, 1958; Gabriel, 1968 ; Aoki and Yanase, 1970; Domnas and McInnis, 1974; ~~Bobyn~~ Brobyn and Wilding, 1968 77). In those studies where histochemical tests have been performed the pigment has been

identified as melanin (Gabriel, 1968 ; Gotz and Vey, 1974). The phenomenon is not confined to the surrounding cuticle since the hyphal wall may become heavily melanised (Nyhlen and Unestam, 1980) or enveloped by a melanotic capsule (Gotz and Vey, 1974). However there appears to be few instances where melanization prevents invasion. An apparent exception is the extreme blackening of the mosquito, *Culiseta inornata*, cuticle under appressoria of *Coelomomyces psorophorae* which correlated both with a failure to penetrate and disintegration of the young hyphae. Conversely when penetration did occur only a mild reaction was seen (Travland, 1979).

Defensive reaction to fungal invasion within insect haemolymph are common. These may take the form of phagocytosis and encapsulation by haemocytes and humoral encapsulation. The mechanisms involved have been reviewed by Salt (1970), Whitecomb *et al*. (1974) and Gotz and Vey (1974). Although many of the *Deuteromycotina* initiate a wide-spread active response by host haemocytes, this may not prevent rapid infection (Hurpin and Vago, 1958), and strain specificity may affect the outcome. Fargues *et al*.(1976) found that an injected inoculum of 10 conidia of species-specific strain of *M. anisopliae* killed larvae of the beetle *Orycetes rhinoceros* or *Cetonia auratia*, but the minimum lethal dose was 10^4 conidia per larva in inappropriate combinations even though haemocytes of *O. rhinoceros* encapsulated the conidia of each strain equally 24 h. However, after 4 days, whilst the non-adapted strain was still encapsulated, the adapted strain had grown out from the

enveloping haemocytes, possibly aided by an anti-haemocytic toxin, the existence of which has been shown *in vitro* by Vey and Quiot (1975).

The extensive haemocytic response to infection by Deuteromycete fungi (*M. anisopliae, B. bassiana, A. flavus*), is not always equalled with virulent entomopathogens of the Zygomycotina and Mastigomycotina. Although Klein and Coppel (1973) observed encapsulation of *Entomophthora tenthredinis* by agglomerated blood cells in the sawfly, *Diprion similis,* Brobyn and Wilding (1977) found no agglomeration of *E. aphidis* in *Aphis pisum.* Similarly, whereas crayfish haemocytes readily encapsulate hyphae of *Aphanomyces astaci in vitro* (Unestam and Nylund, 1972), another member of the *Mastigomycotina, Coelomomyces punctatus,* is not attacked by haemocytes of the mosquito, *Anopheles quadrimaculatus* (Powell, 1976).

The ability of the host to respond may depend in part, on whether or not protoplasts form the parasitic stage of the fungus (Roberts and Humber, 1981). Haemocytes of the eastern hemlock looper, *Lambdina fiscellaria fiscellaria,* adhered strongly to hyphae and hyphal bodies of *Entomophthora egressa in vitro* but not to protoplasts either *in vivo* or *in vitro* (Dunphy and Nolan, 1980). Protoplasts are part of the natural life cycle of *E. egressa* in grasshoppers (MacLeod, Tyrell and Welton, 1980). These structures are also produced during the early stages of mycosis in *A. quadrimaculatus* by *C. punctatus* (Powell, 1976).

Three strategies may be employed for the use of entomopathogenic fungi to control insect pests (Roberts and Humber, 1981). A fungal disease may be established within a pest population to keep numbers below a threshold by releasing an inoculum of a virulent isolate. Alternatively, the fungus can be used as a microbial insecticide with large scale applications of the fungus each time the pest population exceeds its economic threshold. Finally they may be used within an integrated control programme. This approach has much to commend it as, amongst other advantages, dual applications of insecticide and fungus may prove synergistic (Roberts and Yendol, 1971). Insects, in common with other animals, are more susceptible to disease when under stress (Steinhaus, 1958) and there is much evidence that chemical insecticides can act as stressors promoting mycosis (Benz, 1971). Dual applications may provide two advantages. On the one hand they may permit the use of reduced doses of both insecticide and fungus (Roberts and Humber, 1981) while on the other they may reduce the likelihood of resistance developing to either of the two agents (Bowman, 1980). An increased number of sites of action will reduce the likelihood of favourable mutations conferring resistance.

When dual applications are made, combinations are usually chosen empirically and co-operation between fungus and insecticide is likely to be gratuitous. By contrast, diflubenzuron, an inhibitor of chitin synthesis in insects (Post and Vincent 1973; Introduction to Chapter 1), could *a priori*, act as a "true synergist", facilitating entry of pathogenic fungi by

weakening insect cuticle. The present study was done to test this hypothesis, using the pathogenicity of the green muscardine fungus *(Metarhizium anisopliae)* for the tobacco hornworm moth *(Manduca sexta)*.

CHAPTER 1

Effect of diflubenzuron on larvae of *Manduca sexta*

Introduction

Diflubenzuron is a 1-(4-chlorophenyl)-3-(2,6-difluoro-benzoyl) urea, which has potent insecticidal properties. A formulation of this compound is marketed by Philips-Duphar B.V. under the trade name Dimilin*. It is recommended particularly for use against Lepidopteron pests, but it also has larvicidal activity against species of Coleoptera, Diptera and Orthoptera (Mulder and Gijswijt, 1973; Ishaaya and Casida, 1974; Ker, 1977).

The mode of action of this insecticide is very different from conventional chemical insecticides in that Dimilin does not cause death of an insect directly, rather death is the result of a failure to moult successfully. Dimilin acts by interfering with cuticle formation. In most insects the bulk of larval cuticle is laid down during the intermoult period (post-ecdysial cuticle) e.g. over 60% in larvae of Elaterids (Zacharuk, 1972), 80% in *Rhodnius prolixus* (Wigglesworth, 1957) and 90% of the endocuticle in *Calpodes elothius* (Locke, 1965). Therefore applying Dimilin during this time severely reduces the strength of the cuticle. If treatment occurs during

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* Hereafter diflubenzuron will be referred to as Dimilin, because the formulated product was employed in the present work, see Materials and Methods of this chapter.

the production of the next larval cuticle (pre-eddysial cuticle), the new cuticle will be weakened and poorly attached to the epidermal layer. As a result the cuticle cannot withstand the internal pressure and the muscular movement at the time of ecdysis. Consequent upon this, the insect fails to shed the exuvia and dies from water loss through splits in the new cuticle.

The mode of action of Dimilin has been a matter for some debate. Hypotheses include: interference with ecdysone metabolism (Yu and Terriere, 1977); enhanced chitinase production (Ishaaya and Casida, 1974); inhibition of chitin synthetase (Deul *et al.*, 1978); production of short chain chitin (Clarke *et al.* , 1977) and deformation of epidermal cell microvilli (Grosscurt, 1978). Some of these hypotheses are not mutually exclusive.

The ability of Dimilin to reduce cuticle formation *in vivo*, in the absence of hormones tends to discount an effect on ecdysone metabolism (Mitsui *et al.*, 1980), while its speed of action (fully active 80 minutes after injection (Ker, 1978)) mitigates against enhancement of chitinase production (Deul *et al.*, 1978). Post *et al.* (1974) found that uridine diphosphate-N-acetylglucosamine accumulated in Dimilin treated larvae of *Pieris brassicae*. They suggested that Dimilin therefore prevented the polymerization of chitin by blocking chitin synthetase. Support for the chitin synthetase inhibition hypothesis comes from the failure to extract chitin from Dimilin-

treated cuticle of *Locusta migratoria* (Hunter and Vincent, 1974). However, the method employed by Hunter and Vincent for the estimation of chitin is not specific(by differential weighing before and after treatment to remove protein). If Dimilin caused the production of short chitin chains (Clarke *et al.*, 1977) failure to detect chitin could have been due to its dispersion in the alkaline medium used to extract the protein (Ker, 1978).

Following an appraisal of the studies attempting to estimate chitin content of Dimilin-affected cuticle, Ker (1978) concluded that chitin secretion might well be less than 10%, but was unlikely to be zero. In contrast,chitin synthesis in locust peritrophic membrane was 70% of normal (Clarke *et al.*, 1977). The latter result is consistent with the idea of Dimilin being a competitive inhibitor of chitin synthetase. However, one must conclude that the overall mode of action of Dimilin in this system is different to that in integumentary cuticle formation.

It is rather disconcerting for the chitin synthetase inhibition scheme that Dimilin had no effect on a cell free extract of chitin synthetase of *Mamestra brassicae* (Mitsui *et al.*, 1981). The scheme would still be tenable if, *in vivo,* Dimilin acted through its metabolites (Mitsui *et al.*, 1981). However Ivie and co-workers have shown Dimilin to be relatively resistant to metabolism and its metabolites have no effect on chitin biosynthesis (Ivie and Wright, 1978; Ivie and Bull, 1980).

Perhaps Grosscurt's (1978) proposal that Dimilin acts directly on the epidermal cell should receive further consideration.

Ker (1978) has pointed out that other factors could magnify even a small inhibitory effect of Dimilin on chitin synthetase activity. Of particular interest is the fact that in fungi, chitin microfibrils are synthesised as units by a multi-enzyme granule (Ruiz-Herrera *et al.*, 1975). Ker suggested that if a similar system operates in insects then the blockage of a single enzyme site in a granule could disrupt the whole granule.

In some regions of insects no stable layer of cuticle is deposited under Dimilin-treatment, suggesting that without chitin, proteins are incapable of forming a distinct layer. This is true of much of the larval cuticle of *Pieris brassicae* (Mulder and Gijswijt, 1973), pharate adult cuticle of *Schistocerca gregaria* and the intersegmental membranes of *Locusta migratoria* (Ker, 1978). However, stable Dimilin affected cuticle is deposited in the tergites and sternites of adult *Locusta migratoria* and the prealar arm, hind tibia and pharate mandibles of adult *Schistocerca gregaria* (Ker, 1977, 1978). It could be that either the protein is particularly stable in these regions, a small amount of chitin is sufficient for stability in these particular areas, or tanning plays a significant role (Ker, 1978). However, it should be noted that there is no evidence that Dimilin inhibits synthesis of integ-

umental proteins (Salama *et al.*, 1976).

Dimilin is known to act mainly as stomach poison (Mulder and Gijswijt, 1973; Neal, 1974; Wright, 1974; Retnakaran and Smith, 1975; Audemard, 1978) because of its poor penetrability of insect cuticle (Neal, 1974). Nancy *et al.* (1978) found little contact activity of Dimilin against *Choristoneura occidentalis* and *Orgyia pseudotsugata*. However, moderate contact toxicity has been shown against *Boarmia selenaria* (Ascher, 1978), while Ascher and Nemny (1976) found that Dimilin had comparable topical and stomach poison activities against *Spodoptera littoralis*. Dimilin also shows useful contact activity against insect eggs (Ascher and Nemny, 1974; Ascher *et al.*, 1978 a, b). This ovicidal effect may result either from direct exposure or indirectly by application to a gravid female (Grosscurt, 1978).

Mitsui *et al.* (1980) demonstrated that Dimilin had insecticidal activity against fifth instar larvae of *Manduca sexta*. However, to the author's knowledge there is no other published data on the effects of Dimilin on *Manduca sexta*. Therefore the work described in this chapter was done in order to:

1. Design a protocol for testing the activity of Dimilin against young larvae of *Manduca*.
2. Determine contact and stomach poison ED50s of Dimilin against young larvae for use in trials with *Metarhizium anisopliae* (see Chapter 3).

Materials and Methods

Diflubenzuron, formulated as "Dimilin" in a 25% wettable powder, was a gift from Philips-Duphar B.V. Crop Protection Division, The Netherlands.

1. Use of Dimilin as stomach poison

Treatment was started 6h ± 6h after insects had ecdysed to 1st, 2nd, 3rd or 4th instar as appropriate.

i. Application to artificial diet

Appropriate dilution series of the insecticide were made in distilled water and each concentration (recorded as % active ingredient (a.i.) w/v; see Busvine, 1971) was kept agitated on a magnetic stirrer prior to use, to prevent settling out of the suspension. Small blocks (ca. 1.5 cm^3) of diet were briefly dipped in the suspension then allowed to drain of excess liquid before transfer to compartments of an ice-cube tray or small Petri dishes (50 mm). The trays were covered with glass plates to stop desiccation of the food. Control blocks were dipped in distilled water. Larvae were placed singly on a block of treated diet and allowed to feed for 48 hours. The insecticide treated diet was then replaced with normal diet till 48 h post-ecdysis.

Experimental insects were maintained either (a) in a constant temperature room (25 ± 2^{o}C) and a low relative humidity

(50 - 60%) for those kept in compartments of ice-cube trays or (b) in plastic boxes (5 litre capacity) lined with wet cotton wool to give a high relative humidity (100%). The plastic boxes were maintained in an environmental cabinet (Fisons) set at 25 ± 1°C and 90% RH. Under 17 h light: 7 h dark photo-period.

ii. Application to tomato leaves

Tomato leaves were briefly dipped in suspensions of Dimilin (see 1(i) above) and left to air dry at room temperature. Insects were placed in individual Petri dishes (50 mm) with a piece of tomato leaf and allowed to feed for 48 h. The insecticide treated food was then replaced with a normal tomato leaf until 48 h post ecdysis. Insects were maintained as in 1(i)b above.

iii. Spraying whole tomato plants to run off

Tomato plants were grown either in trays of 6 plants each or singly in pots. When the plants reached a height of 20 - 25 cm they were sprayed with a series of Dimilin suspensions to run off using an aerosol (Fisons), air dried at room temperature and then inoculated with second instar larvae (6 h ± 6 h). Trays were kept in small glass house (3 m^2), and the pots were stored in an environmental cabinet (see 1(i) above.

A gap of ca. 10 cm was left between trays to prevent movement of insects between trays. Insects were observed for 2 h after application to the plants, to ensure that they anchored and started to feed.

The relative humidity was boosted to 60 - 80% by showering the gravel floor twice a day. The temperature ranged between 20 - 40°C. A photoperiod of 17 h light: 7 h dark was maintained.

Plants in the environmental cabinet were inoculated with insects, caged with a polythene bag(perforated for gas exchange) and supported with three fine canes. The conditions within the environmental cabinet are similar to those of 1(i) b above except for the relative humidity which was set at 70%.

The number of live insects was counted 5 days after the start of the experiment.

2. Use of Dimilin as a contact poison

Insects either 6 h ± 6 h or 42 h ± 6 h after the previous moult, were sprayed to run off with Dimilin suspensions using an aerosol (Fisons). They were allowed to air dry at room temperature before being confined with artificial diet. Insects were maintained as described in 1(i) a or 1(i) b.

3. Criteria of death

Those insects were considered dead which:-

(i) stopped feeding prematurely and failed to respond to an irritating stimulus viz. died before the moult.

(ii) Attempted to ecdyse but failed.

(iii) Failed to feed for 48 h after ecdysis.

Mortality within an experimental group was recorded as

% premoult death (= all insects in (i) above)

% failed to ecdyse (= insects in (i) and (ii))

% mortality (= insects in (i) + (ii) + (iii))

4 . Measurementof cast headcapsules

Attempts to measure the width of exuvial headcapsules with a vernier screw gauge failed because the material was too brittle. However, thelength of cast headcapsules were determined using this method. Statistical comparisons of the data were done using the ANOVA test as described by Gomez (1968), where appropriate, reference was made to the statistical tables of Fisher and Yates (1963). Values of $p < 0.05$ were taken as being significant. The level of significance of the difference between treatment and control means was determined using the least significant difference test (LSD) (Gomez, 1968).

5. Measurement of faecal production

Faecal pellets from 5 insects were collected over the 48 h

period of treatment with Dimilin, placed in an aluminium foil container, oven dried for 24 h at 100°C and then weighed to the nearest mg.

Statistical comparisons of data were performed in a similar manner to 4 above.

6. Statistical analysis of insecticidal bioassays

The results were subjected to probit analysis (Finney, 1952) using the maximum-likelihood programme (Ross, 1970) from the programme library of Rothamsted Station, Harpenden, England.

7. Maintenance of the stock culture of *Manduca sexta*

The adults were kept in wooden framed boxes covered with muslin (1 m^3), each accommodated 12 individuals. Under 50 ± 10% relative humidity, 26 ± 2°C, and a 12 h "bright" light: 12 h "dim" light photoperiod. This unusual photoperiodic regime was used to enable adults to feed and mate. Adults were maintained on an artificial yellow flower containing a cotton wool wick impregnated with a 10% sucrose solution. A tobacco plant was maintained within the cage box as a site for oviposition and the eggs were collected once a day, and placed in a plastic cup which was covered and kept at 26°C within an incubator.

The larvae were reared on a modification of Bell and Joachim's artificial diet (Bell and Joachim, 1976) (see Appendix 1) at 50 ± 10% RH and 25°C under a long day (17 h light; 7 h dark).

Results

Symptoms of insecticide poisoning

Dimilin applied as either a contact or stomach poison brought about similar symptoms in larvae of the tobacco hornworm *Manduca sexta*. Insects were seemingly unaffected until reaching apolysis stage. Those under high dose died at a pre-ecdyse stage while others initiated ecdysis by getting rid of the old headcapsule exuvia and the old skin got stuck somewhere downwards, especially at the abdominal segments. Due to its weakness the underneath skin usually broke which was followed by loss of some body fluid, then the insect turned black and died. The broken site was usually at the thoracic segments. Some insects were trapped to partially shed skin and starved to death without loss of body fluid. Also insects seemed to dehydrate when maintained under low relative humidity. Besides that, some insects succeeded to moult but failed to feed. This is the general phenomenon under effect of contact poison when insects were maintained at high relative humidity and part of those insects showed fluid droplets on the external surface of the cuticle. Those succeeding to moult and failing to feed usually starved to death.

1. Use of Dimilin as a stomach poison

(i) Application to artificial diet

(a) In an environment with a low relative humidity

These experiments were conducted on first, second, third and fourth instar larvae. The data are recorded in Tables

1A - 4A of Appendix 2. Table 1 summarises the results of the probit analysis. A linear relationship is apparent between the probit transformed % mortality/% failed to ecdyse data and log$_{10}$ of the insecticide concentrations, for first, second and third larval instars. The data for the fourth larval instar did not fit the probit equation and ~~was~~ were discarded. Chi^2 tests show a good fit of points about lines at the 5% level of significance.

The % mortality ED50 of the first instar larvae was 1.5 fold more than that of second and third instars, but there was little difference between % mortality ED50s of second and third instars. % mortality ED50 and % failure to ecdyse ED50 were substantially the same for second and third instars; few insects died from starvation after a successful ecdysis.

Cast head capsules from third and fourth instars larvae (ecdysed to fourth and fifth instars respectively) treated with Dimilin, were significantly smaller than controls (see Tables 2 - 7).

(b) In an environment with a high relative humidity

The effect of high relative humidity on the susceptibility of first and second instar larvae to Dimilin was determined. The data are recorded in full in Tables (5A - 7A) of Appendix 2. Table 8 summaries the results of the probit analysis. Second instar larvae were 5-fold more susceptible

to Dimilin than first instar. Among the second instar, % mortality ED50 < % failure to ecdyse ED50 < % premoult death ED50. This might have been expected, expressing the result another way, the greater the dose the earlier the death. The slopes of the probit lines were uniformly high and there was little heterogeneity except for % failure to ecdyse of the second instar.

(ii) Applied to tomato leaves under a high relative humidity

A series of experiments was done to test the effects of Dimilin on second instar larvae, when the insecticice was applied to tomato leaves under high relative humidity. This was necessary because dual applications of fungus and insecticide had to be performed under high relative humidity using natural food for the insects (see Chapters 2 and 3). In addition to recording % mortality, % failure to ecdyse and % premoult death, the faecal pellets were collected as a measure of food consumption.

The data of the bioassay are recorded in full in Tables (8A and 9A) of Appendix 2. Table 9 summaries the results of the probit analysis. As in the last series of experiments (1(i)b) % mortality ED50 < % failure to ecdyse ED50 < % premoult death ED50. Once again this simply reflects the fact that the greater the dose the earlier the kill. The slopes of the probit lines were considerably less than in the comparable experiments when insects were fed on artificial diet (1(i)b).

Faecal pellet production was significantly reduced in insects dosed with 0.0035% a.i. (w/v) Dimilin or more with respect to the control (Tables 10 - 12).

(iii) Spraying whole tomato plants, experiments performed in an environment with a low relative humidity

The data are recorded in full in Tables (10A - 12A). of Appendix 2. Table 13 summarises the results of the probit analysis. The % mortality ED50 was similar in glasshouse and environmental cabinet. However, the slopes were considerably larger than those of the comparable experiments done using detached tomato leaves (see 1(ii)).

2. Use of Dimilin as a contact poison

(a) In an environment with a low relative humidity

The data are recorded in full in Tables (13A - 19A) of Appendix 2. Table 14 summarises the results of the probit analysis. The % mortality ED50s of young (6 h ± 6 h) first, second and third instar larvae ~~was~~ were similar. Old larvae (42 h ± 6 h) of both first and second instars were more susceptible than young larvae (6 h ± 6 h). The slopes of the probit lines were smaller for older than for young larvae. Chi^2 tests revealed no significant heterogeneity except for % failure to ecdyse data of the old first instar larvae.

Exuvial head capsules from old (42 h ± 6 h) Dimilin-treated

second instar larvae (ecdysis to third instar) were not significantly different from controls. However, cast head capsules from young (6 h ± 6 h) fourth instar larvae (ecdysis to fifth instar) were significnatly smaller than the controls ($p < 0.01$) (Tables 15 - 19).

(b) In an environment with a high relative humidity

The data are recorded in full in Tables (20A and 21A) of Appendix 2. Table 20 summarises the results of the probit analysis. In contrast to the situation under low relative humidity (see 2(a)), % mortality ED50 for young (6 h ± 6 h) second instar larvae was 2.6-fold less than that for first instar larvae. The slopes of the probit lines were high and there was no heterogeneity.

Summary of the Results

1. ED50 of Dimilin against first instar larvae was similar under low and high humidity regimes; as stomach poison.
2. Second instar larvae were more susceptible to the insecticide at high RH than low RH, whether applied as stomach or contact poison
3. At high RH second instar larvae were more susceptible to Dimilin when fed on tomato leaves than when fed on artificial diet.
4. Dimilin was 3-5 fold (depending on stage - first, second, and third instar tested) less effective as a contact poison

than as a stomach poison in an environment with a low RH.

5. The slopes of the probit analyses graphs from dipping experiments were steeper when experimental insects were fed on artificial diet than when fed on tomato leaves.

6. Exuvial head capsules of young (6 h ± 6 h) insects treated with a high dose of Dimilin (applied topically or with the food) were significantly smaller than those of the controls. This phenomenon was not observed if the treatment was started on older insects (42 h ± 6 h).

7. Dimilin treated insects produced significantly less faecal material during the 48 h of treatment than controls.

8. First instar larvae were less susceptible than second instar larvae to a stomach dose of Dimilin. However, first , second and third instar larvae were equally affected by a contact dose of the insecticide (low RH regime).

9. Old (42h ± 6 h) first and second instar larvae were more susceptible to the contact action of Dimilin than young (6 h ± 6 h) first and second instar larvae.

10. Greater proportions of deaths occurred after rather than during, ecdysis where insects were maintained at high RH than when held at low RH. This trend was apparent in insects dosed topically or *per os*.

Table 1. Effect of Dimilin on first, second and third instar larvae of *Manduca sexta*; insecticide applied as a stomach poison to artificial diet, insects maintained at low RH

Insect stage	ED50 and 95% fiducial limits % ai (w/v) Dimilin	Slope ± SE	Chi^2	Degrees of freedom	Table number in Appendix 2
First†	.0042 (.0036-.0049)	2.97 ± .347	1.050	3	1A
Second†	.0026 (.0013-.0036)	2.72 ± .717	1.631	2	2A
Second*	.0030 (.0016-.0040)	2.62 ± .660	0.786	2	2A
Third†	.0027 (.006-.0046)	1.64 ± .438	0.748	3	3A
Third*	.0039 (.0017-.0059)	1.83 ± .423	1.009	3	3A

† ED50 for % mortality

* ED50 for % failure to ecdyse

Table 2. Effect of Dimilin on shed exuvial head capsules of third instar larvae of *Manduca sexta* insecticide applied as a stomach poison to artificial diet, insects maintained at low RH

Concentration of Dimilin % ai. (w/v)	No. of insects	Mean ± SE exuvial head capsule size (mm)
0.062	19	2.16 ± 0.021
0.031	17	2.18 ± 0.034
0.015	16	2.27 ± 0.029
0.007	14	2.28 ± 0.03
0.0035	19	2.30 ± 0.025
0.0	15	2.27 ± 0.043

Table 3. Analysis of variance of the data from Table 2.

Source	SS	df	MS	Observed F	Tabulated F 5%	Tabulated F 1%
Treatment	0.19825	5	0.0396	2.43*	2.33	3.24
Residual	1.53135	94	0.0162909			
Total	1.7296	99				

* Significant at 5% level of significance

Table 4. LSD test for comparisons between pairs of treatment and control from Table 2

Concentration of Dimilin % ai (w/v)	Concentration response mean expressed in mm	Concentration mean - control mean: Observed	Concentration mean - control mean: Expected at 5% level
0.062	2.16	0.11*	0.08601
0.031	2.18	0.09*	0.0886
0.015	2.27	0.00 NS	0.0913
0.007	2.28	0.01 NS	0.0929
0.0035	2.30	0.03 NS	0.0864
0.0	2.27		

* Significantly different from the control at the 5% level

NS not significantly different from the control

Table 5. Effect of Dimilin on shed exuvial head capsules of fourth instar larvae of *Manduca sexta*; insecticide applied as a stomach poison to artificial diet - insects maintained at low RH

Concentration of Dimilin % ai (w/v)	Number of insects	Mean ± SE exuvial head capsule size (mm)
0.062	11	3.69 ± 0.11
0.031	10	3.8 ± 0.09
0.015	13	3.99 ± 0.8
0.007	11	3.95 ± 0.5
0.0035	13	3.98 ± 0.04
0.0	10	4.08 ± 0.8

Table 6. Analysis of variance of the data from Table 5

Source	SS	df	MS	Observed F	Tabulated F 5%	Tabulated F 1%
Treatment	1.1126	5	0.22252	3.73**	2.36	3.32
Residual	3.9386	66	0.5968			
Total	5.0512	67				

** significant at 1% level of significance

Table 7. LSD test for comparisons between pairs of treatment and control from Table 5.

Concentration of Dimilin % ai (w/v)	Concentration response mean expressed in (mm)	Concentration response mean - control response mean: Observed	Expected at 5%; 1% level
0.062	3.69	0.39 **	0.209; 0.249
0.031	3.80	0.28 **	0.214; 0.254
0.015	3.99	0.09 NS	0.201
0.007	3.95	0.13 NS	0.209
0.0035	3.98	0.10 NS	0.201
0.0	4.08		

** Significantly different from the control at the 1% level

NS not significantly different from the control

Table 8. Effect of Dimilin on first and second instar larvae of *Manduca sexta*; insecticide applied as a stomach poison to artificial diet, insects maintained at high RH

Insect stage	ED50 and 95% fiducial limits % ai (w/v) Dimilin	Slope ± SE		Chi^2	Degrees of freedom	Table No. in Appendix 2
First†	.0047(.0035-.0061)	2.83	.490	3.83	3	5A
Second†	.00099(.0008-.0012)	2.46	.280	1.38	3	7A
Second*	.0018(.0015-.0021)	2.28	.226	11.15∂	3	7A
Second§	.0089(.0075-.0102)	2.67	.217	5.44	3	6A

† ED50 for % mortality

* ED50 for % failure to ecdyse

§ ED50 for % pre-moult death

∂ significant heterogeneity at 0.05 level

Table 9. Effect of Dimilin on second instar larvae of *Manduca sexta*; insecticide applied as a stomach poison to tomato leaves, insects maintained at high RH.

Insect stage	ED50 and 95% fiducial limits % ai (w/v) Dimilin	Slope ± SE		Chi^2	Degrees of freedom	Table number in Appendix 2
Second†	.00059(.0003-.0008)	1.60	.246	6.87	3	9A
Second*	.0012(.0008-.0019)	1.30	.194	9.07∂	3	9A
Second§	.0054(.0041-.0069)	1.52	.164	4.20	3	8A

† ED50 for % mortality

* ED50 for % failure to ecdyse

§ ED50 for %pre-moult death

∂ significant heterogeneity at 0.05 level

Table 10. Effect of Dimilin on faecal pellets production of second instar larvae of *Manduca sexta*, insecticide applied as a stomach poison to tomato leaves, insects maintained at high relative humidity

Concentration of Dimilin % ai (w/v)	Number of replicates	Mean weight of faecal pellets ± SE (mg)	
0.031	4	24.25	1.25
0.015	4	24.00	0.71
0.007	4	24.75	1.25
0.0035	4	25.75	0.74
0.0018	4	27.25	1.25
0.0	4	29.75	1.11

Table 11. Analysis of variance of data in Table 10

Source	SS	df	MS	Observed F	Tabulated F 5%	Tabulated F 1%
Treatment	83.9	5	16.78	3.5*	2.77	4.25
Residual	85.7	18	4.761			
Total	169.6	23				

* Significant at 5% level of significance

Table 12. LSD test for comparison between pairs of treatment and control from Table 10

Concentration of Dimilin % ai (w/v)	Concentration response mean expressed in (mg)	Concentration response mean - control response mean: Observed	Expected at 5%; 1% level
0.031	24.25	5.50**	$LSD_{(.05)}$=3.24
0.015	24.00	5.75**	
0.007	24.75	5.00**	$LSD_{(.01)}$=4.44
0.0035	25.75	4.00*	
0.0018	27.25	2.50 NS	
0.0	29.75		

** significantly different from the control at the 1% level

* significantly different from the control at the 5% level

NS not significantly different from the control.

Table 13. Effect of Dimilin on second instar larvae of *Manduca sexta*; insecticide sprayed as a stomach poison to tomato plants, insects maintained at low RH

Maintained at:	ED50 and 95% fiducial limits % ai (w/v) Dimilin	Slope ± SE	Chi^2	Degrees of freedom	Table No. in Appendix 2
Glasshouse second†	.0014 (.0012-.0016)	2.59	.889	3	11A
Environmental cabinet second†	.00126 (.001-.0015)	2.89	3.97	3	12A

† ED50 for % mortality

Table 14. Effect of Dimilin on first, second and third insect larvae of *Manduca sexta*; insecticide applied as a contact poison - insects maintained on artificial diet at low RH

Insect stage	ED50 and 95% fiducial limits % ai (w/v) Dimilin	Slope ± SE	Chi^2	Degrees of freedom	Table No. in Appendix 2
First†	.012(.0095-.014)	3.24 ± .760	1.018	3	14A
First*	.0035(.0005-.0068)	1.73 ± .333	11.65§	3	15A
Second†	.0125(.0097-.0166)	1.92 ± .317	.725	3	16A
Second*	.0069(.0043-.0095)	1.79 ± .256	5.805	3	17A
Third†	.013(.0098-.0174)	2.55 ± .419	3.12	3	18A

† ED50 for % mortality of 6 h ± 6 h insects

* ED50 for % mortality of 42 h ± 6 h insects.

§ Significant heterogeneity at the 0.05 level.

Table 15. Effect of Dimilin on shed exuvial headcapsules of second instar larvae (42 h ± 6 h) of *Manduca sexta*; insecticide applied as a contact poison, insects maintained on artificial diet at low RH

Concentration of Dimilin % ai (w/v)	Number of insects	Mean ± SE exuvial head capsule size (mm)
0.031	18	1.45 ± .02
0.015	21	1.45 ± .01
0.007	21	1.44 ± .02
0.0035	22	1.47 ± .02
0.0	23	1.47 ± .02

Table 16. Analysis of variance of data from Table 15

Source	SS	df	MS	Observed F	Tabulated F at 5% level
Treatment	0.01090	4	0.002725	0.41 NS	2.32
Residual	0.66872	100	0.0066872		
Total	0.67962	104			

NS there is no significant difference between treatment means.

Table 17. Effect of Dimilin on shed exuvial head capsules of fourth instar larvae (6h ± 6 h) of *Manduca sexta*; insecticide applied as a contact poison, insects maintained on artificial diet at low RH

Concentration of Dimilin % ai (w/v)	Number of insects	Mean ± SE exuvial head capsule size (mm)
0.62	13	3.81 .04
0.31	10	3.76 .05
0.062	13	3.94 .04
0.031	11	3.98 0.03
0.015	12	3.99 0.03
0.0	14	4.04 .04

Table 18. Analysis of variance of data from Table 17

Source	SS	df	MS	Observed F	Tabulated F 5%	Tabulated F 1%
Treatment	0.7367	5	0.14734	7.94**	2.36	3.32
Residual	1.2439	67				
Total	1.9806	72				

** significant at 1% level of significance

Table 19. LSD test for comparisons between pairs of treatment and control from Table 17

Concentration of Dimilin % ai (w/v)	Concentration response mean expressed in (mm)	Concentration response mean - control response mean: Observed	Expected at 5%; 1% level
0.62	3.81	0.23**	0.103; 0.122
0.31	3.76	0.28**	0.111; 0.131
0.062	3.94	0.10 NS	0.103
0.031	3.98	0.06 NS	0.107
0.015	3.99	0.05 NS	0.105
0.0	4.04		

** Significantly different from the control at the 1% level

NS not significantly different from the control.

Table 20. Effect of Dimilin on first and second instar larvae of *Manduca sexta*; insecticide applied as a contact poison - insects maintained on artificial diet at high RH

Insect stage	ED50 and 95% fiducial limits % ai (w/v) Dimilin	Slope ± SE		chi^2	Degrees of freedom	Table Number in Appendix 2
First†	.0164(.0135-.0212)	3.57	.825	3.61	3	20A
Second†	.00632(.0055-.0072)	3.75	.392	1.46	3	21A

† ED50 for % mortality

Discussion

The symptoms of Dimilin poisoning in *Manduca* larvae were similar to those described for other insects(Mulder and Gijswijt, 1973; Ascher and Nemny, 1976; Salama *et al.*, 1976; Salama and Magd -Eldin, 1977; Mitsui *et al.*, 1980; Reed and Bass,1980; Abd-Allah and Ralph, 1981). Characteristically insects failed to shed the exuvia during ecdysis. However, at high doses death occurred pre-ecdysially due to a direct toxic action of the insecticide on the larvae and there were no external symtpoms, while at low doses a successful moult was followed by failure to feed. The lack of external symptoms in pre-moult young *Manduca* larvae dosed with Dimilin (this chapter) is in stark contrast to the ruptured intersegmental membranes and abdominal bloating observed in Dimilin treated 5th instar larvae (Mitsui *et al.*, 1980). 90% of larval growth occurs during the 5th larval instar (Williams, 1980), placing a particular premium on normal post-ecdysial growth of the cuticle during this stage. When experimental insects were maintained under a high humidity, a greater proportion of deaths at all doses occurred after ecdysis. This observation is consistent with the view that the primary cause of death in Dimilin-treated insects is dehydration through splits in the new cuticle (Mulder and Gijswijt, 1973), since high RH would reduce water loss (Loveridge, 1968) and allow more insects to complete ecdysis. Death of many of the insects that successfully-moulted was,however, only postponed, as they appeared unable to feed. Abd-Allah and Ralph (1981) have attributed a similar condition in Dimilin-treated insects of *Lymantria dispar* (=*Porthetria*) to weakened mouth parts.

First instar larvae were less susceptible to a stomach dose of Dimilin than second ~~isntar~~ instar larvae, under high or low humidity. A similar reduction in sensitivity to insecticide with age has been reported before (Salama and Magd-Eldin, 1977; Retnakaran *et al.*, 1980; Eliott and Anderson, 1982). Beevi and Dale (1980) suggested that the later instars of *Spodoptera mauritia* were more susceptible to Dimilin because they consumed proportionally more Dimilin-treated food than first instars. A similar mechanism could be operating in the present case. Since first, second and third instar *Manduca* larvae were equally susceptible to a topical dose of Dimilin, it would certainly seem unlikely ~~to~~ that the first instar was inherently more resistant to the insecticide than older larvae. On the other hand, second instar larvae were more susceptible to Dimilin under a high than a low relative humidity, where, as first instar were not.

Older first and second instar larvae proved more susceptible than their younger counterparts to the contact action of Dimilin. Hopkins and Chamberlain (1978) found a similar situation in the louse *Bovicola limbatus*, where susceptibility of the older larvae to Dimilin was connected with the time of greatest weight gain. However, the optimum period for Dimilin administration is during synthesis of the next cuticle. All insects stop feeding prior to ecdysis (Wigglesworth, 1972) therefore constrains on the success of the stomach action of Dimilin are the time lag between cessation of feeding and cuticle production, and the half-life of the insecticide in the insect. Both factors are limiting in fifth instar desert locusts (Ker, 1977) but apparently

not in first and second instar *Manduca* larvae.

It is generally believed that RH has little effect on toxicity of insecticides (Beard, 1958), although Gaines and Mistric (1958) reported a reduction in the activity of DDT pyrethrum, toxaphene and calcium arsenate at high RH,towards house flies, *Dendrolimus* larvae and boll weevils. However, in the present work Dimilin was considerably more toxic to larvae of *Manduca sexta* under high rather than low RH. Grosscurt (1978) found a similar reduction with larvae of *Leptinotarsa decemlineata*. The reason for this is not immediately apparent but the phenomenon is a positive advantage in the present context because dual application of Dimilin and fungus must take place in an environment at high RH, to ensure successful invasion of the insect by the fungus (see Chapters 2 and 3).

Manduca larvae were some 1.5 fold more susceptible to Dimilin when fed on tomato leaves than when fed on artificial diet (insecticide applied by dipping, environment with high RH). Although variations in the susceptibility of insects to insecticides dependent on diet have been reported before (Gaines and Mistric 1958; Wood *et al.*, 1981) in the present case the variation could be more illusory than real. In that tomato leaves could ~~have~~ have adsorbed more insecticide than the diet when dipped into Dimilin suspensions. Setting aside chemical concentrations the surface area/volume ratio of tomato leaves is greater than that of the artificial diet. In a similar vein, the smaller slopes of log dose/probit response graphs in experiments

where insects were fed tomato leaves than in those experiments where they were fed artificial diet may simply be due to the more uneven distribution of the insecticide on the surface of the leaves (Hoskins and Craig, 1963). However, the slopes of probit lines were high in experiments where tomato plants were sprayed with Dimilin suspensions.

Although Dimilin was considerably more toxic towards *Manduca* larvae when administered orally than when applied topically, supporting work on other insects (Mulder and Gijswijt, 1973; Ascher *et al.*, 1978; Gillette *et al.*, 1978), Dimilin has significant ~~stomach~~ contact activity. Mitsui *et al.* (1980) have also shown that Dimilin has insecticidal activity against 5th instar *Manduca* larvae, when applied topically or incorporated in the diet. They recorded a total mortality LD50 of 0.5 µg/larva for a topical dose. Interestingly, pre-ecdysial mortality was 20 - 30% irrespective of the topical dose. Mulder and Gijswijt (1973) argued that phenylbenzoylurea insecticides were merely stomach poisons and could not penetrate insect cuticle. Accordingly Gillette *et al.* (1978) concluded that topical toxicity of Dimilin against larvae of *Choristoneura occidentalis* and *Orgyia pseudotsugata* was an artifact due to contamination of the diet from Dimilin on the cuticle. This is difficult to refute but old first and second instar *Manduca* larvae were more susceptible to the contact action of Dimilin than young insects which fed more.

The headcapsule of *Manduca* larvae appears to be one of the

few structures that does not increase in size during post-ecdysial growth (Nijhout,1975) though Williams (1980) found an increase in chitosan content of headcapsules cuticle from fifth instar *Manduca* during the course of the instar, and concluded that the cuticle of the headcapsule thickened post-ecdysially. ~~THerefore~~ Therefore it is strange that cast headcapsules from larvae treated with Dimilin early in the instar were significantly smaller than those from controls.

Dimilin has a disruptive effect on post-ecdysial abdominal cuticle, but it does not stop synthesis (see Chapter 4). In contrast if it is assumed that Dimilin stops the synthesis of post-ecdysial cephalic cuticle, then, at ecdysis, Dimilin affected headcapsule cuticle will be considerably thinner than normal. Similarly because much of the normal cephalic cuticle (pre and post ecdysial) is highly tanned and therefore not hydrolysed prior to ecdysis, the exuvial headcapsule from normal insects will be thicker than that from Dimilin-treated larvae. On the basis of this hypothesis the reason for the exuvial headcapsules of Dimilin-treated insects being significantly shorter than those of control insects would be that the thinner Dimilin-treated cuticle shrinks more after ecdysis. Treatment of larvae in the second half of instar occurred too late to significantly influence post-ecdysial cuticle and hence the size of the exuvial headcapsule.

A stomach dose of Dimilin which was enough to kill more than 80% of insects reduced the amount of food consumed during the

48 h of treatment possibly due to an anti-feedant effect. This is at odds with the findings of Mulder and Gijswijt (1973) who failed to detect any effect of Dimilin on feeding in a number of insect species. However, Reed and Bass (1981) did find a reduction in the quantity of food eaten by *Pseudoplusia includens* under treatment with Dimilin.

CHAPTER 2

Effect of four species of entomopathogenic fungi on larvae of *Manduca sexta*

Introduction

Fungal diseases are prevalent in most of the orders of the class Insecta, particularly in Lepidoptera, Diptera, Coleoptera, Hemiptera (aphids and scale insects) and Hymenoptera (bees). Therefore it is presumably more than a coincidence that pests of medical importance such as lice, fleas, tsetse flies and cockroaches appear relatively free from mycoses; the reason for this remains to be established (Roberts and Yendol, 1971).

Epizootics caused by entomopathogenic fungi are relatively common, though this has only been realised comparatively recently (Ferron, 1978). Wilding and his colleagues (Wilding, 1970; 1975; Wilding and Lauckner, 1974), have established that *Entomophthora* sp. can provide a degree of natural control of aphids in grain crops in favourable years. However, the value of this control is doubtful because it is unpredictable (dependent on many variables) and often too late to be of any economic significance. Attempts to improve the efficiency of endemic entomopathogenic fungi by changing agronomic practices to optimise environmental conditions has received little attention (Ferron, 1978; Ignoffo, 1978).

However the alternative, to introduce inocula artificially, has been the subject of much study (see for example, reviews by

Roberts and Yendol, 1971; Ferron, 1978; Roberts and Humber, 1981). The last named strategy may consist of either releasing diseased insects or broadcasting a suitable fungal propagule. The biggest successes have been achieved using the latter method (Hall and Burges, 1979; Roberts and Humber, 1981). This is perhaps not surprising because *in vitro* fungal production is much cheaper and easier than *in vivo*. Many attempts have been made to use artificial inocula to control insect pests on a small scale but there are realtively few cases where production has been on a commercial scale and application has been over a wide area (Weiser, 1982). Most of these appear to have been done with Deuteromycte fungi and the details of some of the important cases are given in Table 21.

There are a number of reasons why entomopathogenic fungi have not become widely available as "off-the-shelf pesticidal agents" for use as an integral part of a crop protectionists armoury. The problem areas include: a correct blend of environmental conditions (particularly high relative humidity) is necessary for the development and spread of disease; the difficulty in producing a virulent, stable preparation on a large scale (Ferron, 1978; Weiser, 1982). In addition susceptibility to fungicides complicates the process of fitting entomopathogenic fungi into an integrated control programme (Hall and Dunn, 1958; Ignoffo *et al.*, 1975). However, there is general if not universal compatibility with insecticides (Jaques and Morris, 1980).

Despite these problems there are many advantages to the use of parasitic fungi for the control of insect pests. These add credibility to the continued research into this form of biological pest control. Not the least of these advantages is the cost of development and registration which is only a fraction of that required for a chemical pesticide (Lisansky, pers. comm). and the apparently low health hazards to man, vertebrates in general, and other natural enemies of pest insects (Ferron, 1978; Austwick, 1979). Additional factors include the general susceptibility of all developmental stages (viz. not confined to a feeding stage as fungi usually penetrate their hosts externally) and speed of action (at least with respect to other forms of microbiological control (Deacon, 1983). For insects, e.g. aphids entomopathogenic fungi are the only viable forms of microbiological control, because of the absence of other endemic disease causing organisms (Deacon, 1983). Unfortunately fungi of e.g. Entomophthorales are specialised parasites which are difficult to culture *in vitro* and their spores are very susceptible to desiccation (Roberts and Humber, 1981; Wilding, 1980).

Possible new avenues of approach for the use of parasitic fungi for pest control are: selection of hyper-virulent mutants (a considerable amount of work has already been done on this in the USSR (Pavlyushin, 1978)); use of lures to attract insects to an inoculum source; dual application of insecticides and fungi (already tried in Eastern Europe, but neglected in the West, see Chapter 3). The object of the work described in this thesis was to investigate the last named of these possibilities (see Chapter 3).

To the author's knowledge there are no records of the test organism, the tobacco hornworm *Manduca sexta*, suffering from a fungal disease. Therefore it was necessary to screen a number of candidate fungi for pathogenicity towards *Manduca*. The strains tested were simply those which were available within the laboratory. However, insects may acquire immunity to endemic fungal diseases and thus the most virulent pathogens may have obscure origins (Lappa, 1978).

Table 21. Examples of large scale production of entomopathogenic fungi

Fungus	Product	Country (Company if applicable)	Insect species	Reference
Aschersonia aleyrodis	-	USSR	*Dialeurodes*	Weiser (1982)
Beauveria bassiana	Boverin	USSR	*Leptino tarsa decemlineata*	Ferron (1978)
Beauveria bassiana	-		*Ostrinia nubilalis*	Hussey and Tinsley(1980)
Metarhizium anisopliae	Metaquino	Brazil	Spittlebugs	Ferron (1980)
Metarhizium anisopliae	-	Apia, Western Samoa	*Oryctes rhinoceros*	Roberts and Humber(1981)
Verticillium lecanii	Vertilac	Tate and Lyle U.K.	Aphids	Hall (1982)
Hirsutella thompsonii	Mycotal Mycar	" USA	Whitefly Mites	Weiser (1982)

Materials and Methods

Table 22 shows the sources of the isolates of the entomopathogenic fungi screened.

Media and Preparation of Media

Sabouraud's dextrose agar (SDA) was routinely used for fungal cultivation. It consists of 1% peptone, 2% agar and 4% dextrose. The dry ingredients were thoroughly mixed and then one litre of a distilled water was added. The ingredients were encouraged to dissolve by placing the beaker in a hot water bath. The viscous solution was then transferred to medical flasks (300 ml) for autoclaving under 15 psi for 15 minutes at 121°C.

When cultivating the fungus *Nomuraea rileyi* the above medium was made up containing in addition 1% yeast extract (Wayne *et al.*, 1977). Flasks cooled to ca. 40°C were poured in 9 cm sterilized plastic petri dishes.

Harvesting Conidia and Viability Tests

Conidia were inoculated on SDA in disposable 9 cm diameter plastic petri dishes (Sterilin Northern Media Labs.) by streaking with a needle loop. The cultures were grown at 27°C for *Metarhizium anisopliae* and 23°C for *Beauveria bassiana*, *Nomuraea rileyi* and *Verticillium lecanii* in the dark. Sporulation occurred within a week for *B. bassiana*, *M. anisopliae* and *V. lecanii*, and within 2-3 weeks for

Table 22. Sources of the isolates of the entomopathogenic fungi screened

Species	Insect of origin	Donor
Metarhizium anisopliae	*Curculio caryae*	Roberts
Verticillium lecanii	*Macrosiphoniella sanborni*	Tate and Lyle
Beauveria bassiana	*Curculio caryae*	Tate and Lyle
Nomuraea rileyi	*Spodoptera exempta*	CMI*

* Commonwealth Mycological Institute

N. rileyi. After sporulation the cultures were kept in a fridge at 4°C for use within six weeks. Conidia of *B. bassiana*, *M. anisopliae* and *N. rileyi* were harvested by flooding the plate with an aqueous solution of 0.4%.Tween 80, while conidia of *V. lecanii* were harvested in sterile distilled water only. Conidia were suspended in the bathing medium by agitating with a glass rod. Then the conidial suspension was decanted, transferred to a waring blender and mixed for 2 - 3 minutes prior to sieving through a double layer of sterilized muslin to remove hyphal fragments. Nutrients carried over from the culture medium and endogenous germination inhibitors were removed by washing (Veen, 1968). This was achieved as follows: The suspension was centrifuged for 3 minutes at 3000 rpm in an MSE bench centrifuge. The supernatant was

decanted and replaced with an aliquot of the washing solution (0.4% T.80 or sterile distilled water (SDW)). Conidia were then resuspended in the washing solution using a vortex mixer (Fisons). This procedure was repeated twice. The concentrations of the harvested conidia were determined using an advance hemocytometer (Weber Scientific International Ltd., England).

Viability tests were carried out routinely by placing an aliquot of nutrient broth containing 10,000 conidia into a sterile cavity microscope slide mounted on a U-shaped glass rod in Petri dishes lined with moist filter paper. Slides were stored either at 27°C or 23°C (as appropriate for the fungus, see above) for 18 - 24 hours. Two drops of 0.5% cotton blue in lactophenol were then added to the suspension to stain the fungi and stop further germination. The preparations were observed down a microscope and the % germination of 200 conidia was recorded from each of 3 - 4 slides. Germination was considered to have occurred when the germ tube was equal in length to the conidium. A batch of conidia was not used unless germination was in excess of 95%.

Application of conidia to larvae of *M. sexta*

1. Crawling

Insects were allowed to crawl on sporulating cultures of fungi for 15 minutes then transferred to Petri dishes, 50 mm

(Sterilin Northern Media Laboratories). Experimental insects were maintained in glass "desiccators' of 5 litres capacity. These were maintained at 100% relative humidity, RH (determined by hygrometer) by lining the bottom half with 15 g absorbant cotton wool moistened with 200 ml of distilled water. Petri dishes containing the insects were supported in the upper half of the desiccator by means of a metal grid. The two halves were sealed with grease (MS Silicone stop lock grease - Edwards - UK) and a ventilation hole in the lid was kept open. The whole lot was stored at room temperature under 17L : 7D photoperiod.

The insects were reared on artificial diet both before and during the experiments.

2. Dipping

The source of the fungus employed in these experiments was the cadaver of a fifth instar *Manduca sexta* which had died from an infection of *M. anisopliae*. In order to produce an axenic culture of *M. anisopliae*, conidia were transferred from the surface of the cadaver to SDA containing the antibiotics chloramphenicol and cycloheximide to suppress the growth of bacteria and saprophytic fungi (Veen, 1968). Condia were harvested from this axenic culture as described above.

Insects were reared on artificial diet during the first larval instar. Following the moult to the second instar, they were dipped briefly into a suspension of conidia in 0.4% Tween 80 then transferred to 50 mm Petri dishes containing tomato leaves. These were maintained in a desiccator (see 1 above). The whole lot was stored in an environmental cabinet set at 70 - 90% RH: 25°C and 17L : 7D photoperiod. Fresh tomato foliage was provided every 48 hours.

3. Injection

The method of injection was essentially that described by Locke and Condoulis (1966). Insects were anaesthetised by immersion in distilled water for 5 - 10 minutes. Suspensions of conidia in 0.4% Tween 80 were injected through the second proleg using a 5 µl microsyringe (SGE Australia Ltd.) Individuals which bled from the injection site were discarded. Insects were kept either in plastic tea cups or Petri dishes (according to size) and placed in a moist chamber (see 1 above). The feeding regime was as described in 1 above.

4. Spraying

Insects were sprayed with a conidial suspension in a similar manner to that used in the experiments with Dimilin (see Materials and Methods, Chapter 1). Control insects were sprayed with 0.4% Tween 80 solution. Insects were reared on artificial diet without formalin, sorbic

acid and methyl-p-hydroxybenzoate for 48 hours from the start of the experiment, prior to transfer to diet containing these antibiotics.

Assessment of Mortality

The timing of the assessment of mortality and the criteria of death were evolved during the course of the preliminary screen (see Results section). In subsequent experiments insects were considered dead which:

(i) stopped feeding prematurely and failed to respond to an irritating stimulus viz prodding with a blunt needle

(ii) attempted to ecdyse but failed

(iii) failed to feed for 48 h after ecdysis.

Cadavers were transferred to Petri dishes which were lined with moistened filter paper and kept at 27°C or 23°C (see "harvesting and viability tests of conidia" above) for a period of two days to allow the fungus to break through the insect integument and sporulate on the surface. If conidia of the appropriate fungus appeared on the surface of the insect it was assumed to have died from mycosis. Extending the period of incubation beyond 48 h did not increase the incidence of fungal sporulation on cadavers of experimental insects. No growth of saprophytic fungi occurred on bodies of control experimental insects.

In order to assess whether false positives could have resulted from sporulation due to non-penetrant hyphae on the surface of the insect, preliminary trials were carried out in which the cadavers were surface sterilized by dipping in 70% ethanol for 1 minute and washed twice with (SDW) for 5 minutes each prior to being placed in moistened Petri dishes. This procedure did not significantly alter the proportion of insects scored as being killed by mycosis. Therefore surface sterilization of cadavers was dispensed with.

Mortality within an experimental group was recorded as

% premoult death (all insects in (i) above)

% mortality (= insects in (i) and (ii) and (iii))

% mycosis

Effect of antifungal compounds on germination of conidia of *Metarhizium anisopliae in vitro*

The compounds formalin, methyl-p-hydroxybenzoate and sorbic acid are known to have antifungal activity (Ignoffo *et al.*, 1977). In order to test the effects of these compounds on *M. anisopliae*, conidia were suspended in nutrient broth containing X0.25, X1 or X4 the dose of the antifungal compounds to be used in the artificial diet (see Appendix 1). An aliquot of 25 µl of the suspension (400,000 conidia/ml) was placed on a cavity microscope slide. 3 - 4 Slides were prepared per treatment and stored and evaluated in a manner described above (see harvesting of conidia and viability tests).

Statistical Analysis of Bioassays

The results were subjected to probit analysis (Finney, 1952) using the maximum-liklihood programme (Ross, 1977) from the programme library of Rothamsted station, Harpenden, England.

Results

Preliminary screen for an entomogenous fungus with pathogenicity for *Manduca sexta*.

1. Response of *Manduca* to crawling over sporulating cultures of *Beauveria bassiana, Metarhizium anisopliae, Nomuraea rileyi* and *Verticillium lecanii*.

No infection occurred in initial experiments using any of the four fungi. Subsequently it was realised that some of the chemicals in the artificial diet (see Appendix 1) had antifungal activity, viz. formalin, sorbic acid and methyl-p-hydroxybenzoate, (Ignoffo *et al.*, 1977: Ignoffo and Garcia, 1978), which could have been responsible for the apparent lack of virulence. Support for this came from an *in vitro* experiment. Each of the three compounds caused 100% inhibition of germination of *M. anisopliae* at the concentration used in the artificial diet (Table 23).

In the light of the above, an experiment was done to compare the effect of the four species of fungi on *Manduca* when the insect was reared on a diet with or without the addition of antifungal compounds.

Little infection occurred when insects were allowed to crawl over sporulating cultures of the four fungi if the insects were subsequently reared on diet containing the antifungal compounds (table 24). In contrast when these

Table 23. Effect of antifungal compounds on germination of conidia of *Metarhizium anisopliae in vitro*

	Control	Formalin			Sorbic acid			Methyl-p-hyroxy-benzoate		
		*XO.25	X1	X4	XO.25	X1	X4	XO.25	X1	X4
% germination	92	8.5	O	O	O	O	O	O	O	O

* Concentration of compounds employed was either XO.25, X1 or X4 of that normally present in the artificial diet (see Appendix 1).

Table 24. Response of *Manduca* to crawling over sporulating cultures of *B. bassiana*, *M. anisopliae*, *N. rileyi* and *V. lecanii*

Insect stage (instars)	Number of insects per treatment	Diet (A) with or (NA) without antifungal compounds	Time of assessment of Mortality - days after dosing	RESPONSE Treatment % Mortality	Treatment % Mycosis	Control % Mortality	Control % Mycosis
M. anisopliae							
1	40	A	10	25	0	13	0
2	34	A	7	27	21	4	0
3	15	A	7	13	13	0	0
2	50	NA I	5	100	100	24	0
2	65	NA II	5	91	90	0	0
B. bassiana							
2	40	NA I	5	100	95	24	0
2	65	NA II	5	97	90	0	0
V. lecanii							
3	10	A	5	0	0	0	0
2	40	NA I	5	55	5	24	0
N. rileyi							
2	65	NA II	5	19	13	0	0

I Insects fed on diet without antifungal compounds from day 1 of the first instar to the end of the experiment.
II Insects fed on diet without antifungal compounds for a 48 h period from the time of dosing with the fungus.

substances were excluded from the food 100% mortality was achieved with *M. anisopliae* and *B. bassiana* and more than 50% with *V. lecanii*. However the diet was invaded by *Aspergillus* sp. and control mortality was in excess of 20%. Indeed only 5% of the mortality in *V. lecanii* treatment could be attributed to the entomopathogenic fungus directly. The timing of the removal of the antifungal chemicals was not critical, and the isolates of *N. rileyi* and *V. lecanii* employed had little pathogenicity towards *Manduca* and were discarded.

During the course of this preliminary screen experimental insects were kept for an extended period to determine if and when death occurred. First instar larvae, dosed with *M. anisopliae* were followed for 10 days, this being the normal interval between first and fourth instars. Mortality during this period was 25%, no insects died from mycosis and control death was unusually high (13%). In all other inoculated groups, though insects were kept for 5 - 7 days, deaths only occurred, due to mycosis, prior to the first moult following inoculation (see Table 24). Note that the second and third instars last ca. 3 days each.

Therefore a protocol was devised for assessing mortality which took into account the fact that if death was going to occur it would happen prior to or during ecdysis (see Materials and Methods). As a safeguard an additional criterion of death was included viz. those failing to feed for 48 h after the

moult. Insects normally started feeding 12 h after ecdysis and no insects recovered from a 48 h self imposed period of starvation.

2. Comparisons between the pathogenicity of *M. anisopliae* and *B. bassiana* towards second instar larvae of *Manduca sexta* - inoculum applied by spraying

This experiment was done to test the performance of the above mentioned fungi, against *Manduca* using a more refined method of application. Insects were reared on artificial diet without antifungal compounds for a 48 h period from the time of dosing with the fungus. *Metarhizium anisopliae* proved to be considerably more pathogenic than *Beauveria bassiana* to *Manduca* (see Table 25). Accordingly, *M. anisopliae* was chosen for further investigation.

3. The effect of the mode of application on the pathogenicity of *M. anisopliae* against *Manduca* larvae

Metarhizium anisopliae was selected for further trials using different methods of application. Experimental insects were maintained on tomato leaves to obviate the problems encountered with the artificial diet (see above).

Table 25. Comparison between the pathogenicities of *M. anisopliae* and *B. bassiana* towards second instar *Manduca sexta*, Inoculum applied by spraying.

Treatment conidia/ml	number of insects	% mortality* mean ± SE	% mycosis* mean ± SE
M. anisopliae 1.25×10^8	80	81 ± 2.4	78 ± 3.3
B. bassiana 1.38×10^8	80	24 ± 5.6	16 ± 3.8
M. anisopliae 9×10^7	60	57 ± 4.4	45 ± 5.0
B. bassiana 2.35×10^8	60	2 ± 1.7	2 ± 1.7
Control	140	0	0

* Mean of 4 replicates each of 15 - 20 insects.

A. Topical application

An arithmetic dilution series of *M. anisopliae* was made up in 0.4% aqueous Tween 80. Second instar larvae (6h ± 6h) were dipped into suspensions and the % mortality recorded in Tables (1A-5A) of Appendix 3. Table 26 summarises the results of the probit analysis of % mortality, % mycosis and % premoult death. 1×10^5 conidia/ml produced ca. 50% mortality and the majority of deaths was due to mycosis. Most insects died prior to the moult.

B. Injection

Third instar larvae (6h ± 6h) were considerably more susceptible to an injected dose of *M. anisopliae* than second instar larvae were to a topical dose. The results are shown in Tables (6A and 7A) of Appendix 3. Table 27 summarises the results of the probit analysis. 11 - 120 conidia/larva were sufficient to kill ca. 50% of larvae.

Pathology of the disease caused by *M. anisopliae*

The symptoms of disease were recorded from insects of second, third, fourth and fifth instars which were either injected with or dipped in conidial suspensions.

Infected insects stopped feeding within 48 h of inoculation, indicated by a failure to produce faecal pellets. This was the most consistent early symptom of infection. Subsequently insects

Table 26. Effect of dipping second instar larvae (6h ± 6h) of *Manduca sexta* in conidial suspensions of *Metarhizium anisopliae*: insects maintained on tomato leaves at high relative humidity

Assay number	ED50 and 95% fiducial limits $x10^5$ conidia/ml	Slope ± SE	Chi^2	Degrees of freedom	Table number in Appendix 3
1	†0.71(.225-1.285)	1.1 ± .2	7.70	4	2A
	*0.49(.23-1.323)	0.61 ± .17	1.60	4	
	§1.41(.693-2.155)	1.14 ± .18	11.37 ¶	4	
2	†1.137(.83-1.532)	2.03 ± .26	0.44	3	3A
	*2.09(1.55-2.813)	2.06 ± .25	3.30	3	
	§2.61(1.78-3.63)	1.79 ± .26	1.91	2	
3	†1.11(.75-1.61)	1.36 ± .16	7.18	3	4A
	*1.39(.98-1.97)	1.53 ± .18	5.92	3	
	§3.54(.257-4.86)	1.85 ± .24	2.81	2	
4	†1.15(.63-2.24)	1.37 ± .33	0.12	2	5A

† % mortality
* % mycosis
§ % pre-moult death

¶ The dose-response line shows a significant heterogeneity at the 5% level. All other assays have a good fit of points about the lines at the 5% level.

Table 27. Effect of an Intrahaemocoelic injection of conidia on third instar larvae of *Manduca sexta*

Assay number	LD50 and 95% fiducial limits: conidia/larva	Slope ± SE	Chi^2	Degrees of freedom	Table no. in Appendix 3
1	120 (24 - 455)	0.77 .19	1.28	3	6A
2	11 (0 - 110)	0.37 .15	0.51	2	7A

became sluggish and the time of death depended on the size of the dose (for criteria of death, see Materials and Methods).

Within 48 h of injection melanic deposits occurred in the cuticle in the vicinity of the injection site (not observed with controls). Areas of brown pigmentation were also found in second instar larvae dipped in suspension of conidia, 3 days after inoculation.The extent of the melanization was positively correlated with the size of the inoculum, and was not localised. In those dosed insects which managed to ecdyse successfully melanic deposits were also present within the new cuticle. Indeed brown pigmentation was most pronounced in the new cuticle just after the moult. Riddiford and Hori (1981) found that phenoloxidase was normally present in new *Manduca* cuticle within a few hours of ecdysis.

A melanic reaction within the integument is a common symptom of mycosis in insects, particularly in Lepidoptera e.g. *Heliothis zea* (Lefebvre, 1934); *Pieris brassicae* (Tanada, 1955) and Bombyx mori (Aoki and Yanase, 1970).

Second instar larvae dipped in high concentrations of conidia (8×10^6/ml) did not show any melanization. Instead, after 3 days the posterior part of the insect turned yellow, the old cuticle (presumptive exuvial cuticle) came away in flakes and the abdominal contents broke through the new cuticle underneath.

These alternative symptoms at high doses have been recorded before (Tanada, 1955; Madelin, 1963; Aoki and Yanase, 1970). It is possible that the absence of melanic deposits during acute infection was due to death occurring prior to the normal time period of phenoloxidase production. In other words during the early part of an instar *Manduca* larvae are not competent to respond to a fungal infection by producing melanin.

Little growth of the fungus occurred within *Manduca* prior to death. A batch of fourth instar larvae were dipped into a suspension of 10^8 conidia/ml and several insects were sacrificed and dissected 24, 48 and 72 h after inoculation. The only obvious sign of pathogenesis were changes in colour of certain organs. The Malpighian tubules turned dark brown, the nervous system and fat body turned yellow. Few hyphal bodies were found in the haemocoel or within squashed preparations up to 72 h post inoculation.

When death occurred the insect body was soon transformed into a stiff cadaver. If the last named was maintained in a moist warm environment, mycelia of the fungus emerged through spiracles, intersegmental membranes and sites of injection, and within 2 days the cadaver was covered with green conidia. This scenario was true for all developmental stages examined (instars 2 - 5).

The symptoms described above are consistent with the hypothesis that death was caused by insecticidal toxins (Abalis,

1981; Suzuki, 1971). In support of this, it is interesting to note that Samuels (unpublished) has shown that the strain of *M. anisopliae* employed in the present work (M.E.I.) produces destruxins *in vitro*.

Discussion

Artificial diets have the advantage that they are considerably easier to produce than plant foliage. However, they routinely contain antibiotics and preservatives which affect the stability of the pathogens (Burges and Thomson, 1971). Such was the case in the present work, where formalin, methyl-p-hydroxybenzoate and sorbic acid significantly reduced the performance of the entomopathogenic fungi under investigation. Similar results have been recorded by Garcia and Ignoffo (1978), who studied the interaction between *Beauveria bassiana* and *Trichoplusia ni*.

Grula *et al*. (1978) circumvented the problem by leaving the antibiotics out of their diet. Death of *Heliothis zea* from *B. bassiana* occurred in advance of, and thus the experiment was not influenced by, the saprophytic growth of *Aspergillus* sp. and *Penicillium* sp. on the diet. However, in the present work when formalin, methyl-p-hydroxybenzoate and sorbic acid were left out of the diet a high control mortality was correlated with the growth of *Aspergillus* sp. and in subsequent experiments insects were reared on tomato leaves.

The strain of *Metarhizium anisopliae* tested in this chapter proved to be an effective pathogen of *Manduca sexta*. The ED50 (mean of 4 separate experiments) for % mortality was 1×10^5 conidia/ml . This compares favourably with the value of the virulent strain of *Verticillium lecanii* against *Macrosiphoniella sanborni* (ED50 = 10^5 conidia/ml)(Hall, 1976).

The dipping technique employed was sufficiently reproducible to give good agreement for ED50 mortality values between the 4 assays. In addition for most assays and parameters (% mortality % mycosis and % premoult death) there was no significant heterogeneity about dose-response lines.

The slopes of dose-response lines were ca. 1.47 ± .19; 1.4 ± .42; 1.59 ± .23 for % mortality, % mycosis and % premoult death respectively, which are considerably less than that of Hall (1976) (ca. 2.26). Meynell and Meynell (1965) suggested that because microorganisms work independently of each other the slope is unlikely to exceed 2, unless the pathogen produces a toxin (e.g. *Bacillus thuringiensis*). Indirect evidence was presented in the results section of this chapter that implicates a fungal toxin in the cause of death of *Manduca* larvae. If true, then although every effort was made to standardize test insects e.g. age, nutritional status and size, the slopes were somewhat smaller than the theoretical optimum (see review by Burges and Thomson, 1971).

Control mortality using the dipping technique was always low (see Appendix 3). However, % mortality of experimental insects was always greater than % mycosis, No attempt was made in this study to determine causes of death other than from mycosis. However, Zacharuk (1973) has shown that bacteria can accompany invading fungal hyphae into an insect, and thus septicaemia may account for a proportion of experimental deaths.

CHAPTER 3

COMBINED EFFECTS OF DIFLUBENZURON AND *M. ANISOPLIAE* ON LARVAE OF *MANDUCA SEXTA*

Introduction

Insects like other organisms are more vulnerable to infectious diseases when under the stress of malnutrition, crowding, or unfavourable environmental conditions (Steinhaus, 1958). In particular insecticides have been observed to act as stressors resulting in increased incidence of natural microbial infection (Ferron, 1978). As a result there have been a number of attempts to use artificial dual applications of insecticides and microbes, including fungi, to control insect pests (see review by Benz, 1971).

A stressor may of itself not cause mortality but only predispose the insect to infection; the converse can also be true, e.g. reduced doses of parathion and trichloronate caused infection due to *Beauveria tenella* in *Melolontha melolontha* with a dose of conidia that did not produce mycosis on its own (Ferron, 1971). Alternatively both agents may prove fatal when applied singly, yet the combined treatment may have greater effect than the sum of the individual components (Ferron, 1978).

The situation defined in the last paragraph constitutes synergism between stressor (insecticide) and microbe (fungus). However, synergism can alternatively be expressed not as

enhanced mortality but as a shortening of the time needed for kill - "temporal synergism" (Benz, 1971).

It is not axiomatic that chemical insecticides will synergise the effects of a fungal inoculum. Indeed pesticides can inactivate entomopathogens e.g. Ignoffo *et al.* (1975) reported that all fungicides and some insecticides and herbicides registered for use on the soyabean crop were deleterious to *Nomuraea rileyi*. Insecticides and microoorganisms may also act synergistically or antagonistically depending on the concentration. Thus combined applications of an ED 22.5 dose of *B. bassiana* with an ED 0-9 dose of Wafatox resulted in 73.7% kill of *Eurygaster integriceps*, where as the combination of *B. bassiana* (ED 22.5) with Wofatox (ED 46.2) caused only 32.6% mortality (Shekhurina, 1959).

Incompatibility between fungus and insecticide may be indirect. Luckey (1968) demonstrated the principle he termed "hormoligosis" viz the stimulatory effect of non-lethal doses of insecticides on insect growth. Such an effect could theoretically increase an insect's resistance to mycosis (Benz, 1971).

A pesticide may neither interfere directly nor indirectly with the virulence of a pathogen yet still be antagonistic, by interfering with the reproduction of the pathogen. Once again there are no examples of this in insect-fungus interactions but Schnyder and Benz (see Benz, 1971) found that non-lethal doses of derris caused premature death of virus

infected *Zeiraphera diniana*, which drastically reduced virus infection.

Combined applications of entomopathogenic fungi and insecticides as a strategy for pest control has been somewhat neglected in the "West". However, extensive use has been made of dual applications of *B. bassiana* and insecticides in Eastern Europe, particularly the USSR, against a number of insect pests on a variety of crops. Control of *Leptinotarsa decemlineata* has been carried out using 2 kg of "Boverin" (a preparation of *B. bassiana*) at 6 x 10^9 conidia/g with 1/5 the normal dosage of DDT, though more recently there has been a trend towards larger single doses of "Boverin" (Ferron, 1978).

Following a laboratory study Fargues (1973) concluded that there was only a very weak synergistic action between *Beauveria* and DDT against *L. decemlineata* despite the fact that the mortality in the combination was greater than the sum of the mortalities in individual treatments. Sub-lethal (non-toxic) doses of DDT caused the gut of *L. decemlineata* to become permeable to pathogenic intestinal bacteria (Pristavko, 1963) and also reduced the number of haemocytes (Telenga, 1957). The presence of the fungus presumably potentiated the action of the bacteria, though most mortality was due to septicaemia. The remainder of the insects were killed either by the fungus, the toxic action of the insecticide or a combination of septicaemia and muscardine (Fargues, 1973).

It was argued in the general introduction that Dimilin could, *a priori*, act as a true synergist, facilitating entry of pathogenic fungi by weakening insect cuticle. The object of the work in this chapter was to test this hypothesis. Dimilin is considered to be an inhibitor of chitin synthetase (see introduction to Chapter 1) and as such could inhibit chitin synthesis in fungi as well as insects (Sowa and Marks, 1975). Therefore an important aspect of the work was to determine whether Dimilin was inhibitory to the germination and growth of *Metarhizium in vitro*. In his review Benz (1971) concluded that combined application of micro-organism and toxic doses of insecticides rarely produced better than independent synergism (for definition see the Materials and Methods of this chapter). However, since combinations of microbes and non-lethal doses of insecticides may not give consistent results (Benz, 1971), a toxic dose of Dimilin was used in the present work.

Materials and Methods

1. Sensitivity of *Metarhizium*-conidial germination and mycelial growth-to Dimilin

Conidia were harvested from a two weeks old culture grown on SDA as described in Chapter 2.

A. Germination test

In preliminary trials, germination tests were performed by suspending conidia of *Metarhizium anisopliae* in nutrient broth containing Dimilin within cavity slides. The last named were placed on U-shaped glass rods in Petri dishes (9 cm) and maintained at 27°C in an incubator; % germination of 1000 conidia/concentration was assessed after 24 h. Those conidia with a germ tube equal in length to the conidium were assumed to have germinated. However, this method proved unsatisfactory because of difficulty in differentiating between the conidia and insecticidal particles especially at high doses. Therefore the paper disc method similar to that described by Ignoffo *et al.* (1975) was used.

A conidial suspension was diluted to a concentration of 4×10^6 conidia/ml using sterile distilled water with the aid of a hemocytometer. 0.25 ml aliquots were pipetted onto 9 cm Petri dishes containing SDA. The inoculum was spread over the surface using a sterile bent glass rod. The Petri dishes were left open in the cabinet for 30 minutes for the water to evaporate.

An insecticide suspension was made up in sterile distilled water as described in Chapter 1. Each concentration was kept under continuous agitation using a magnetic stirrer. Sterile filter paper bioassay discs (13 mm) were briefly dipped in the insecticidal suspensions and transferred individually to inoculated petri dishes (see above). For the control the discs were dipped similarly in sterile distilled water. The Petri dishes were stored in the dark in an incubator set at 27°C for 48 hours.

The experiment was assessed by measuring the inhibition zones in the *Metarhizium* colony surrounding the bioassay discs using a linear scale placed on the distal side of the bottom half of the Petri dish.

B. Growth test

240 ml batches of SDA were made up in 500 ml conical flasks, autoclaved for 15 minutes at 121°C under 15 psi pressure and then transferred while still hot to the flow cabinet. The flasks were left until they were cool enough to handle (ca. 45°C), and then 10 ml aliquots of Dimilin suspensions added . The latter were made up in the flow cabinet as described in Chapter 1. Care was taken to transfer all the insecticidal suspension. Thorough mixing was ensured by shaking the flask for 1 minute. A control was set up by adding 10 ml of sterile distilled water to a flask instead of the insecticide.

The mixtures were poured out into Petri dishes (9 cm)

and allowed to solidify. Then from each a disc of 12 mm diameter was removed from the centre and replaced with a disc of the same size from a 72 h old culture of *Metarhizium anisopliae* grown on SDA. The Petri dishes were stored in an incubator set at 27°C for 72 h.

The experiment was assessed by measuring the diameter of the fungal colony using a linear scale placed on the distal side of the bottom half of the Petri dish.

Statistical analysis of data was performed using the analysis of variance as described by Gomez (1968). Where appropriate, reference was made to the statistical tables of Fisher and Yates (1963). Values of $p < 0.05$ were taken as being significant.

2. Effect of pre-soaking of conidia on the time scale of germination and appressorial formation *in vivo*

Insects used were fourth larval instar that have been reared on artificial diet before inoculation and fed on tomato leaves during the experimental period (see Materials and Methods Chapter 2). Conidia were harvested from a two week old culture grown on SDA (for method, See Chapter 2). The pre-soaked conidia were prepared as follows: The harvested conidia were diluted with sterile distilled water to give a concentration of $8 - 10 \times 10^6$ conidia/ml and 100 ml of the suspension was

placed in a conical flask (250 ml). It was firmly capped with cotton wool and tin foil and left on a Gallenkamp orbital shaker set at 150 rpm with a constant temperature of 27°C in the dark.

After a period of 20 h presoaking in sterile distilled water, conidia were recovered with centrifugation in a bench centrifuge (MSE) at 3000 rpm for 2 minutes. They were then resuspended in fresh sterile distilled water to a final concentration of 1×10^8 condiia/ml as determined using a hemocytometer. Conidia from another 2 weeks old culture were harvested fresh in sterile distilled water (see Materials and Methods, Chapter 2) and the concentration adjusted to 1×10^8 conidia/ml

Insects were dipped briefly in either presoaked (P) or fresh (F) conidial suspension and then transferred to individual Petri dishes (50 mm). Experimental insects were maintained in an environment of constant high relative humidity within an environmental cabinet set at 25°C and a photoperiod regime of 17 L: 7 D (for details See Materials and Methods, Chapter 2).

Replicates of insect cuticle were prepared 6, 12, and 24 hours after the time of inoculation. The method employed was essentially the same as that used by Birkly and Preece (1981) to remove bacteria off the surface of plant leaves. An insect was laid ventral surface down on approximately 5 cm piece

of adhesive tape (3M autoclave tape 1222). Primal AC-33 (Rohm and Haas U.K. Ltd.) was then painted as a thin layer on a sterile microscope slide. The dorsal surface of the pinioned insect was quickly placed in contact with the layer of primal and fixed in position with the adhesive tape. When the primal was dry the inverted replica was carefully peeled away from the cuticle. Germinating conidia on the surface of the cuticle came away with the primal replica. Staining with cotton blue in Lactophenol provided sufficient contrast for the embedded fungal elements to be observed with the aid of a light microscope.

Germination was recorded when the germ tube was equal in length to the conidium and appressional formation was assumed when the width of the germ tube was equal to or greater than that of conidial capsule (see Fig. 1). The proportion of conidia whose germ tubes produced appressoria were recorded as % appressorial formation.

Statistical analysis of data was performed using Student's t-test as described by Bishop (1969) where appropriate, reference was made to the statistical tables of Fisher and Yates (1963). Values of $p < 0.05$ were taken as being significant.

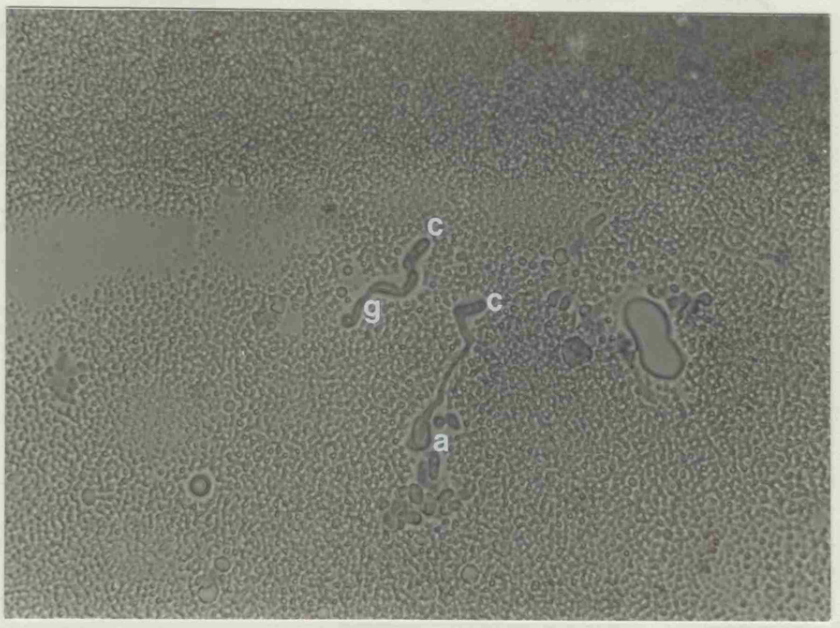


Fig. 1

Primal replica of the surface of Manduca cuticle with "presoaked" germinating conidia attached, 12h after inoculation

Legend: appressorium (a); germ tube (g); conidium (c)

Scale: x 400

3. Effect of pre-soaking of conidia on the pathogenicity of *Metarhizium* towards second instar larvae (6h + 6h) in an environment with a constant high relative humidity.

Insects used were second instar larvae, the method of rearing before and during the experimental period was described previously (see dipping of the Materials and Methods to Chapter 2). Insects were briefly dipped in a conidial suspension and transferred individually to Petri dishes (50 mm), supplied with tomato leaves and stored in an environment with a constant high relative humidity (see the Materials and Methods to Chapter 2). The food was changed after 48 hours

The % mortality was assessed after 5 days and the proportion of deaths due to mycosis was determined two days after transferring the cadavers to moist Petri dishes maintained at 27°C (see the Materials and Methods to Chapter 2).

Statistical analysis of data was performed using a 2 x 2 contingency table as described by Bishop (1969) where appropriate, reference was made to the statistical tables of Fisher and Yates (1963). Values of $p < 0.05$ were taken as being significant.

4. Effect of pre-soaking of conidia on the pathogenicity of *Metarhizium* towards second instar larvae (6h ± 6h) maintained in an environment with a cycling humidity regime.

Second instar larvae were used and treatment was started

6h ± 6h after ecdysis. The maintenance of experimental insects and the method of application of fungal conidia were as described in Section 3 above. Preliminary experiments revealed that less than 24 h of high relative humidity (100%) restricted the efficiency of the fungal pathogen (at a concentration of 2×10^6 conidia/ml mortality was $\leq$ 50% whether fresh or pre-soaked conidia were used). Therefore in this series of experiments insects were maintained either:-

A. for the first 30h post-inoculation under a constant high relative humidity (100%) followed by a further 90 h at 70% relative humidity or

B. for the first 15 h post-inoculation under high relative humidity followed by 9 h under 70% relative humidity, high relative humidity for 15 h and then finally by a period of 81 h at 70% relative humidity (5 day experimental period).

Assessment of the experiment and statistical analysis were similar to those of Section 3 above.

5. Effect of dual applications of Dimilin and *Metarhizium* on second instar larvae in an environment with a constant high relative humidity - agents applied separately

Second instar larvae were used. The maintenance of experimental insects and the method of application of fungal conidia were as described in Section 3 above.

The preparation of suspension of Dimilin and the application of these to tomato leaves were described in Chapter 1 (see Materials and Methods, Section 1(ii)).

Insects were dipped in suspensions of presoaked (P) and fresh (F) conidia at either 6h ± 6h; 30 h ± 6h or 42h ± 6h after ecdysis to second instar (note that second instar lasts for ca. 3 days).

Insects were maintained on insecticide treated food for the first 48 h of the second instar, then fresh untreated tomato foliage was provided for the remainder of the experiment. Control treatments consisted of dipping food and larvae in distilled water. Experimental assessments viz. criteria of death and recording of mortality within an experimental group were similar to those of Chapters 1 and 2.

6. Effects of dual application of Dimilin and *Metarhizium* on second instar larvae in an environment with a cycling relative humidity regime - agents applied separately

These experiments were performed in the manner described in Section 5, with the exception that the insects were maintained under a cycling relative humidity regime (see Section 4B). Experiments assessments were similar to those of Chapters 1 and 2.

7. Effect of dual application of Dimilin and *Metarhizium* on second instar larvae in an environment with a constant high relative humidity - agents applied simultaneously

Experimental insects were second instar larvae (6h ± 6h). They were briefly dipped in either ED50 of conidial suspension, ED50 of Dimilin suspension (contact poison ED50) or combined suspension. The last named was produced by mixing together two equal volumes containing 2 x ED50 of each agent.

Experiments assessments were similar to those of Section 5 above.

Analysis of the data generated in experimental series 5 - 7

Statistical analysis of data from Sections 5-7 was performed using a 2 x 2 contingency table as described by Bishop (1969) where appropriate reference was made to the statistical tables of Fisher and Yates (1963). Values of $p < 0.05$ were taken as being significant. In those experiments where treatments were significantly different ($p < 0.05$), the data was reanalysed using the formula and nomenclature described by Benz (1971) as follows to determine the level of synergism in the dual treatments.

A. Independent Synergism

It was calculated from the formula

$$Pc = Pa + Pb\ (1 - Pa/100)$$

where Pc = expected combined response (e.g. % mortality, % mycosis) for treatments a and b

Pa = expected response of treatment a alone

Pb = expected response of treatment b alone

Independent synergism was deemed to have occurred when the observed combined response was similar to that of the expected.

B. Subadditive Synergism occurred when Pc is greater than independent synergism but less than Pa + Pb.

C. Supplemented Synergism

This occurred when Pc was more than the sum of Pa and Pb.

D. Potentiating Synergism

This occurred when treatment a caused response Pa while treatment b had no effect, i.e. Pb = 0, but the combination response Pc > Pa.

Results

1. Sensitivity of *Metarhizium* - conidial germination and mycelial growth - to Dimilin

A. Germination test

No clear zones of inhibition were seen surrounding the insecticidal discs at any of the concentrations employed. Therefore, it would seem that under the conditions of the experiment, and at the concentrations tested, Dimilin had no effect on germination of *Metarhizium* conidia,

B. Growth test

The results are shown in Table 28. The values are the net growth of mycelial after subtracting the diameter of the original colony disc. The statistical analysis showed no significant differences between treatments at the 5% level (Table 29). Hence it may be concluded that under the experimental conditions and at the concentrations of insecticide employed, Dimilin, had no effect on the growth of *Metarhizium* mycelia.

Table 28. Effect of Dimilin on the growth of *Metarhizium* mycelia *in vitro*

Dimilin % ai w/v	Number of replicates	Mean ± SE diameter of colony (mm)
0	5	12 ± 0.45
0.01	5	12.2 ± 0.37
0.02	5	12.4 ± 0.24
0.04	5	11.2 ± 0.37

Table 29. Analysis of variance of data in Table 28

Source	SS	df	MS	Observed F	Tabulated F 5%
Treatment	4.15	3	1.383	2.05 ns	3.24
Residual	10.8	16	0.675		
Total	14.95	19			

ns = no significant difference between means at 5% level of significance.

2. Effect of pre-soaking of conidia on the time scale of germination and appressorial formation *in vivo*

A recent *in vitro* study has shown that sterile distilled water will not support the germination of *M. anisopliae* conidia (Dillon, unpubl.). However, presoaking of conidia in sterile distilled water for 20 h or more prior to providing a suitable nutrient source significantly reduced the time of germination *in vitro* (Dillon, unpubl.). A series of experiments was designed to determine the effect of presoaking on time to germination and appressorial formation *in vivo*, using the interaction between *M. sexta* and *M. anisopliae*.

The results are displayed in Table 30. It is clear that presoaking of conidia of *M. anisopliae* prior to placing them on the cuticle of *M. sexta* considerably reduced time to germination and appressorial formation with respect to freshly collected conidia. Within 6 h of inoculation 5.8% of the presoaked (P) conidia had germinated but none of the fresh (F) conidia. Within 12 h of inoculation the germination rate reached 69.6% in P conidia when it was only 25% in F conidia. The difference was highly significant ($p < 0.001$). Appressoria were formed within 12 h of inoculation in both treatments. However, there was a 7-fold greater incidence of appressorial formation in P treatment when compared with F fungus; again the difference was highly significant ($p < 0.002$). By 24 h post-inoculation P still displayed considerably greater % germination and incidence of appressorial formation than F ($p < 0.001$).

Table 30. Effect of presoaking of conidia on the time scale of germination and appressorial formation

Treatment	Time of assessment: hours after inoculation	Number of replicas per treatment	Mean number of conidia per replica	Mean ± SE	
				% Germination	% Appressorial formation
Presoaked conidia P	6 h	5	102	5.8 ± 1.1	0 ± 0
	12 h	5	72	69.6 ± 3.1	27.6 ± 4.5
	24 h	5	69	83.6 ± 3.3	45.8 ± 1
Fresh conidia F	6 h	5	61	0	0
	12 h	4	86	25 ± 2.8	3.5 ± 1.3
	24 h	5	102	36.6 ± 3.6	12.4 ± 1.4

3. Effect of pre-soaking of conidia on the pathogenicity of *Metarhizium* towards second instar larvae (6h ± 6h) in an environment with a constant high relative humidity

It was shown in the last section that pre-soaking of conidia greatly reduced the time to germination, *in vivo*. The present experiments were designed to test whether the early conidial germination and appressorial formation brought about enhanced pathogenicity.

A. Concentration of conidia = 0.1×10^6/ml

The results are shown in Table 31. Pre-soaked conidia produced significantly greater mortality than fresh conidia. There was also a greater incidence of mycosis in the larvae dosed with pre-soaked conidia than in those dosed with fresh conidia.

B. Concentration of conidia = 2×10^6/ml

The dose of conidia used in this experiment brought about a large increase in mortality (Table 32) over that observed in Section A above (Table 31). However, it reduced the apparent advantage of the pre-soaked conidia.

4. Effect of pre-soaking of conidia on the pathogenicity of *Metarhizium* towards second instar larvae (6h ± 6h) maintained in an environment with a cycling humidity regime

A restricted period of high humidity must place a premium on speed of germination and penetration, a constraint likely to

Table 31. Effect of pre-soaking of conidia on the pathogenicity of *Metarhizium* to second instar larvae (6h ± 6h) in an environment with a constant high relative humidity - Concentration of conidia = 0.1×10^6/ml

Treatment	Number of insects/ treatment	% mortality	% mycosis
Control	60	10	0
Fresh conidia (F)	60	35	15
Presoaked conidia (P)	60	60	37

Statistical analysis

% mortality	F v P	$p < 0.01$	significant
% mycosis	F v P	$p < 0.01$	significant

Table 32. Effect of pre-soaking of conidia on the pathogenicity of *Metarhizium* to second instar larvae (6h ± 6h) in an environment with a constant high relative humidity - Concentration of conidia = 2×10^6/ml

Treatment	Number of insects per treatment	% mortality	% mycosis	% failing to ecdyse
Control	35	0	0	0
Fresh conidia (F)	40	88	88	86
Presoaked conidia (P)	45	97	97	97

be frequently encountered by entomopathogenic fungi "in the field". The object of these experiments was to determine whether pre-soaking provided conidia with an advantage over fresh conidia when the period of high relative humidity was reduced.

A. 30 h at 100% relative humidity followed by a period at 70% RH to complete a 5 day experimental period

Once again pre-soaked conidia caused a greater mortality than fresh conidia (Table 33). However restricting the period of high humidity to 30 h reduced the pathogenicity of fresh conidia with respect to that under constant high humidity (compare Tables 32 and 33). An even greater loss of performance was noted when the experiment was assessed in terms of % mycosis and % failing to ecdyse.

B. 15 h at 100% RH: 9 h at 70% RH; 15 h at 100% RH followed by a period at 70% RH to complete a 5 day experimental period

The marked difference between the effects of pre-soaked and fresh conidia noted above was lost under the humidity regime used in this experiment (Table 34). % Mortality was considerably less than in experiment A and no significant difference was found between experimental treatments.

Table 33. Effect of pre-soaking of conidia on the pathogenicity of *Metarhizium* to second instar larvae (6h ± 6h); maintained for only 30 h in an environment with a high relative humidity - concentration of conidia = 2×10^6/ml

Treatment	Number of insects per treatment	% mortality	% mycosis	% failing to ecdyse
Control	35	3	0	3
Fresh conidia (F)	40	80	62	60
Presoaked conidia (P)	45	98	98	96

Table 34. Effect of pre-soaking of conidia on the pathogenicity of *Metarhizium* to second instar larvae (6h ± 6h) in an environment with a cycling humidity regime*- concentration of conidia = 2×10^6/ml

Treatment	Number of insects per treatment	% mortality	% mycosis	% failing to ecdyse
Control	60	7	0	3
Fresh conidia (F)	60	67	30	62
Presoaked conidia (P)	60	74	35	55

Statistical analysis

% mortality	F v P	$p \not< 0.50$	not significant
% mycosis	F v P	$p \not< 0.50$	not significant
% failing to ecdyse	F v P	$p \not< 0.80$	not significant

* 15 h at 100% RH: 9 h at 70% RH: 15 h at 100% RH followed by a period to complete 5 days at 70% RH

5. Effect of dual application of Dimilin and *Metarhizium* on second instar larvae in an environment with a constant high relative humidity - agents applied separately

A. 6h ± 6h larvae, concentration of conidia = 0.1×10^6/ml

The results of the experiment are shown in Table 35. All treatments were significantly different from each other apart from DF v DP (% mortality), F v DP(% mycosis) and DF v DP (% failing to ecdyse). Generally dual treatments proved to be significantly more effective than single treatments, using all three forms of assessment. Presoaked conidia caused a significantly greater mortality than fresh conidia, confirming the results of an earlier section (see Table 31). It is also interesting to note that DP caused significantly more mycosis than P, but DF did not result in greater mycosis than F.

The results in Table 36 have been re-analysed using the formula and nomenclature described by Benz (1971) (see Materials and Methods section), to determine the degree of synergism between the insecticide and the fungus (Table 36).Dimilin potentiated the effect of the F fungus in causing mycosis. However, the dual treatment exhibited only weak synergism (subadditive) when the experiment was assessed in terms of % mortality or % failing to ecdyse. A similar situation was found in the experiment using the P fungus apart from the fact that, as assessed by % failing to ecdyse, there was no true synergism (level of synergism = independent).

Table 35. Effect of dual applications of Dimilin and *Metarhizium* on second instar larvae (6h ± 6h) in an environment with a constant high humidity - agents applied separately

Treatment	number of insects	% mortality	% mycocis	% failing to ecdyse
Control	50	4	0	4
Dimilin D	55	55	0	44
Fresh conidia (F)	38	45	29	29
Presoaked conidia (P)	35	51	51	46
DF	36	78	42	67
DP	35	83	71	71

- Concentration of conidia = 0.1×10^6/ml

- Concentration of Dimilin = 0.000625% ai w/v

Statistical analysis

% mortality	D v DF	$p < 0.02$	significant
	D v DP	$p < 0.01$	"
	F v DF	$p < 0.01$	"
	P v DP	$p < 0.01$	"
	DF v DP	$p \geq 0.50$	not significant
% mycosis	F v DF	$p \geq 0.20$	not significant
	P v DP	$p < 0.05$	significant
	DF v DP	$p < 0.01$	"
% failing to ecdyse	D v DF	$p < 0.02$	significant
	D v DP	$p < 0.01$	"
	F v DF	$p < 0.001$	"
	P v DP	$p < 0.02$	"
	DF v DP	$p \geq 0.70$	not significant

Table 36. Results in Table 35 re-analysed to determine the level of synergism, using the system employed by Benz (1971)

Parameter	Control	Expected	Observed	Effect
Dimilin plus fresh conidia DF				
% mortality	4	75	78	Subadditive
% mycosis	0	29	42	Potentiating
% failing to ecdyse	4	60	67	Subadditive
Dimilin plus pre-soaked conidia DP				
% mortality	4	78	83	Subadditive
% mycosis	0	51	71	Potentiating
% failing to ecdyse	4	71	71	Independent

B. 30 h ± 6 h larvae

i. Concentration of conidia = 0.1 x 10^6/ml

The results of this experiment are shown in Table 37. This concentration of conidia was markedly less effective against 30 ± 6h larvae than it was against 6 h ± 6 h insects (compare Tables 35 and 37). Indeed the effects of F conidia were not significantly different from those of the control treatment. Therefore it is not surprising that the dual treatments of insecticide and fungus (F or P) were not significantly different from the Dimilin only treatment, with respect to either % mortality or % failing to ecdyse. Synergism was not tested for.

ii. Concentration of conidia = 2 x 10^6/ml

The concentration of conidia employed in this experiment brought about a substantial mortality in F and P (Table 38). However, combined treatments were not significantly different from single treatments in their effects. The only exception was P v DP. Therefore synergism was not tested for.

iii. Concentration of conidia = 7 x 10^6/ml

The results of the experiment are shown in Table 39. All treatments were significantly different from each other. Therefore dual treatments were more effective than single treatments, in terms of all three assessments. It is also

Table 37. Effect of dual application of Dimilin and *Metarhizium* on second instar larvae (30 h ± 6 h) in an environment with a constant high humidity - agents applied separately

Treatment	Number of insects	% Mortality	% Mycosis	% failing to ecdyse
Control	57	2	0	2
Dimilin D	56	38	0	21
Fresh conidia F	60	5	0	2
Presoaked conidia P	60	10	2	9
DF	58	48	3	20
DP	59	46	3	15

- Concentration of conidia = 0.1×10^6/ml

- Concentration of Dimilin = 0.000625% ai W/V

Statistical analysis

% mortality	D v DF	$p \geq 0.30$	not significant
	D v DP	$p \geq 0.30$	"
% failing to ecdyse	D v DF	$p \geq 0.95$	not significant
	D v DP	$p \geq 0.30$	"

Table 38. Effect of dual application of Dimilin and *Metarhizium* on second instar larvae (30 h± 6 h) in an environment with a constant high relative humidity - agents applied separately

Treatment	Number of insects	% Mortality	% failing to ecdyse
Control	32	6	6
Dimilin	32	48	28
Fresh conidia F	35	43	35
Presoaked conidia P	34	32	23
DF	28	54	27
DP	40	65	39

- Concentration of conidia = 2×10^6/ml

- Concentration of Dimilin = 0.000625% ai w/v

Statistical analysis

% mortality	D v DF	$p \geq 0.50$	not significant
	D v DP	$p \geq 0.10$	"
	F v DF	$p \geq 0.30$	"
	P v DP	$p \leq 0.01$	significant
% failing to ecdyse	D v DF	$p \geq 0.90$	not significant
	D v DP	$p \geq 0.30$	"

interesting to note that pre-soaked conidia applied alone (P) or in combination with Dimilin (DP) were significantly more active against *Manduca* than either fresh conidia (F) or fresh conidia and Dimilin (DF)

The results in Table 39 have been re-analysed using the formula and nomenclature described by Benz (1971), to determine the degree of synergism between the insecticide and the fungus (Table 40). A high degree of synergism occurred whether the fungus was used fresh (F) or pre-soaked (P), in terms of all three assessments.

C. 42 h ± 6 h larvae

i. Concentration of conidia = 2×10^6/ml

The results of this experiment are shown in Table 41. The concentration of conidia employed in this experiment produced mycosis in only 3% of the F and P insects. Therefore, it is not surpring that dual treatments, DF and DP, did not differ significantly from the Dimilin alone, with respect to % mortality and % failing to ecdyse, despite the fact that greater mortality occurred in F and P experimental groups than in the controls. Synergism was not tested for.

ii. Concentration of conidia = 7×10^6/ml

42 h ± 6 h old larvae were almost as resistant to a

Table 39. Effect of dual application of Dimilin and *Metarhizium* on second instar larvae (30 h ± 6 h) in an environment with a constant high relative humidity - agents applied separately

Treatment	Number of insects	% Mortality	% Mycosis	% failing to ecdyse
Control	45	0	0	0
Dimilin D	45	44	0	27
Fresh conidia F	50	26	16	18
Presoaked conidia P	49	57	37	49
DF	45	73	62	64
DP	45	88	88	88

- Concentration of conidia = 7×10^6/ml

- Concentration of Dimilin = 0.000625% ai w/v

Statistical analysis

% mortality	D v DF	$p < 0.01$	significant
	D v DP	$p < 0.001$	"
	F v DF	$p < 0.001$	"
	F v DP	$p < 0.001$	"
	DF v DP	$p < 0.05$	"
% mycosis	F v DF	$p < 0.001$	significant
	P v DP	$p < 0.001$	"
	DF v DP	$p < 0.01$	"
% failing to ecdyse	D v DF	$p < 0.001$	significant
	D v DP	$p < 0.001$	"
	F v DF	$p < 0.001$	"
	P v DP	$p < 0.001$	"
	DF v DP	$p < 0.01$	"

Table 40. Results in Table 39 re-analysed to determine the level of synergism, using the system employed by Benz (1971)

Parameter	Control	Expected	Observed	Effect
Dimilin plus fresh conidia DF				
% mortality	0	59	73	Supplemental
% mycosis	0	16	62	Potentiating
% failing to ecdyse	0	40	64	Supplemental
Dimilin plus pre-soaked conidia DP				
% mortality	0	76	88	Subadditive
% mycosis	0	35	88	Potentiating
% failing to ecdyse	0	61	88	Supplemental

Table 41. Effect of dual application of Dimilin and *Metarhizium* on second instar larvae (42 h ± 6 h) in an environment with a constant high relative humidity - agents applied separately

Treatment	Number of insects	% Mortality	% Mycosis	% failing to ecdyse
Control	30	3	0	3
Dimilin D	33	53	0	23
Fresh conidia F	33	14	3	12
Pre-soaked conidia P	34	10	3	10
DF	32	47	0	19
DP	32	53	0	24

- Concentration of conidia = 2×10^6/ml

- Concentration of Dimilin = 0.000625 % ai w/v

Statistical analysis

% mortality	D v DF	$p > 0.90$	not significant
% failing to ecdyse	D v DF	$p > 0.95$	not significant

concentration of 7 x 10^6 conidia/ml as they were to 2 x 10^6 conidia/ml (Table 42). Only 4% of insects dosed with fresh conidia (F) and 10% dosed with presoaked conidia (P) died from mycosis. Although % mortality in the latter two treatments was higher than % mycosis (15% and 13% respectively), there were no significant differences in mortality between Dimilin only and the dual treatments. Incidence of mycosis in DF was significantly greater than F. Synergism was not tested for.

6. Effect of dual application of Dimilin and *Metarhizium* on second instar larvae in an environment with a cycling humidity regime - agents applied separately.

A major constraint on the use of entomopathogenic fungi for the control of insect pests is the requirement for a high relative humidity during spore germination and penetration of the insect (see Introduction to this chapter). The experiments described so far in this chapter show:

1. Synergistic action of Dimilin and *Metarhizium* against second instar *Manduca* larvae in an environment with a constant high relative humidity; in particular synergism was found with 6h ± 6 h larvae using 0.1 x 10^6 conidia/ml and 30 h ± 6 h larvae using 7 x 10^6 conidia/ml.
2. Presoaking of conidia in distilled water for 20 h prior to use enhanced the pathogenicity of *Metarhizium* towards *Manduca*.

Table 42. Effect of dual application of Dimilin and *Metarhizium* on second instar larvae (42 h ± 6 h) in an environment with a constant high relative humidity - agents applied separately

Treatment	Number of insects	% Mortality	% Mycosis	% failing to ecdyse
Control	20	0	0	0
Dimilin D	30	43	0	37
Fresh conidia F	27	15	4	11
Presoaked conidia P	30	13	10	10
DF	27	52	15	33
DP	30	46	7	43

- Concentration of conidia = 7×10^6/ml

- Concentration of Dimilin = 0.000625% ai w/v

Statistical analysis

% mortality	D v DF	$p \geq 0.50$	not significant
	D v DP	$p \geq 0.70$	"
% failing to ecdyse	D v DF	$p \geq 0.95$	not significant
	D v DP	$p \geq 0.30$	"

Therefore it is of interest to determine whether the enhanced effectiveness of the above treatments was reflected in a reduced need for a constant high relative humidity. To this end, experiments were performed under a cycling humidity regime (see Materials and Methods).

A. 6 h ± 6 h larvae, concentration of conidia = 0.1×10^6/ml

The conidia only treatments (F and P) produced substantial mortalities (Table 43), which were similar to those in the comparable experiment under constant high humidity (see Table 35). Fresh conidia (F) appeared to produce less mortality under cycling humidity (33%) than under constant high humidity (45%), presumably because the conditions of the present experiment placed a premium on speed of germination. The combined fungus and insecticide treatments were not significantly different in their effects from the single treatments. Synergism was, therefore, not tested for.

B. 30 h ± 6 h larvae, concentration of conidia = 7×10^6/ml

The results of this experiment are shown in Table 44. All treatments were significantly different from each other apart from FVDF (% mycosis) and FVDF (% failing to ecdyse). Generally dual treatments proved to be significantly more effective than single treatments. Presoaked conidia caused a significantly greater mortality than fresh conidia, confirming the result of

Table 43. Effect of dual application of Dimilin and *Metarhizium* on second instar larvae (6 h ± 6 h) in an environment with a cycling relative humidity regime - agents applied separately

Treatment	Number of insects	% Mortality	% Mycosis	% failing to ecdyse
Control	30	3	0	3
Dimilin D	30	43	0	33
Fresh conidia F	30	33	30	33
Presoaked conidia P	30	50	40	30
DF	30	51	20	27
DP	30	57	40	40

- Concentration of conidia = 0.1×10^6/ml

- Concentration of Dimilin = 0.000625% ai w/v

Statistical analysis

% mortality	D v DF	$p \geq 0.50$	not significant
	D v DP	$p \geq 0.80$	"
	F v DF	$p \geq 0.10$	"
	P v DP	$p \geq 0.50$	"
	DF v DP	$p \geq 0.50$	"
% mycosis	F v DF	$p \geq 0.80$	not significant
	DF v DP	$p < 0.05$	significant
% failing to ecdyse	D v DF	$p \geq 0.90$	not significant
	D v DP	$p \geq 0.50$	"
	F v DF	$p \geq 0.90$	"
	P v DP	$p \geq 0.20$	"
	DF v DP	$p \geq 0.20$	"

Table 44. Effect of dual application of Dimilin and *Metarhizium* on second instar larvae (30 h ± 6 h) in an environment with a cycling relative humidity - agents applied separately

Treatment	number of insects	% Mortality	% Mycosis	% failing to ecdyse
Control	30	0	0	0
Dimilin D	30	37	0	17
Fresh conidia F	30	50	50	47
Presoaked conidia P	30	67	67	67
DF	30	83	70	67
DP	30	97	93	90

- Concentration of conidia = 7×10^6/ml

- Concentration of Dimilin = 0.000625% ai w/v

Statistical analysis

% mortality	D v DF	$p <$	0.001	significant
	D v DP	$p <$	0.001	"
	F v DF	$p <$	0.001	"
	P v DP	$p <$	0.001	"
	DF v DP	$p <$	0.05	"
% mycosis	F v DF	$p >$	0.1	not significant
	P v DP	$p <$	0.01	significant
	DF v DP	$p <$	0.01	"
% failing to ecdyse	D v DF	$p <$	0.001	significant
	D v DP	$p <$	0.001	"
	P v DP	$p >$	0.1	not significant
	P v DP	$p <$	0.02	significant
	DF v DP	$p <$	0.02	"

previous experiments (see tables 33, 35, 39, 43). Similarly DP treatment was significantly more effective in all three assessments than DF. The results of this experiment are essentially similar to those of the comparable experiment under constant high humidity (see Table 39) and suggest that the cycling humidity regime had little effect on any of the treatments. The results in Table 44 have been re-analysed using the formula and nomenclature described by Benz (1971) (see Materials and Methods), to determine the degree of synergism between the insecticide and the fungus (Table 45). Dimilin clearly potentiated the effects of F and P fungal treatments in causing mycosis. In addition, combined fungus/ insecticide treatments exhibited either "subadditive" or "supplemental" synergism in terms of % mortality and % failing to ecdyse. The level of synergism was not as great as that in the comparable experiment under constant humidities (compare Tables 40 and 45).

7. Effect of dual application of Dimilin and *Metarhizium* on second instar larvae in an environment with a constant high humidity - agents applied simultaneously
6 h ± 6 h larvae, concentration of conidia = 0.1×10^6/ml

In this experiment treatment with Dimilin alone produced only 15% mortality, compared with ca. 45% in the other experiments in the current chapter (see Tables 35, 37, 38, 39, 41,42). The reason for this is well established, namely Dimilin is a considerably more potent stomach poison than contact poison

Table 45. Results of Table 44 re-analysed to determine the level of synergism, using the system employed by Benz (1971)

Parameter	Control	Expected	Observed	Effect
Dimilin plus fresh conidia DF				
% mortality	0	69	83	Subadditive
% mycosis	0	50	70	Potentiating
% failing to ecdyse	0	50	67	Supplemental
Dimilin plus pre-soaked conidia DP				
% mortality	0	79	97	Subadditive
% mycosis		67	93	Potentiating
% failing to ecdyse		73	90	Subadditive

(Gillette *et al.*, 1978, see also Chapter 1). Simultaneous application of Dimilin and *Metarhizium* was achieved by dipping the larvae in a suspension containing fungus and insecticide (see Materials and Methods). Single treatments were performed in a similar manner. In the previous experiments Dimilin was applied to the food.

Combined application of Dimilin and *Metarhizium* were not significantly different in effect from *Metarhizium* alone (Table 46). Synergism was not tested for.

Table 46. Effect of dual application of Dimilin and *Metarhizium* on second instar larvae (6 h ± 6 h) in an environment with a constant high relative humidity - agents applied simultaneously

Treatment	Number of insects	% Mortality	% Mycosis	% failing to ecdyse
Control	40	5	0	3
Dimilin D	40	15	0	3
Fresh conidia F	40	43	35	33
DF	40	40	28	30

- Concentration of conidia = 0.1 x 10^6/ml

- Concentration of Dimilin = 0. 00625 % ai w/v

Statistical analysis

% mortality	F v DF	$p \geq 0.95$	not significant
% mycosis	F v DF	$p \geq 0.30$	"
% failing to ecdyse	F v DF	$p \geq 0.95$	"

Discussion

The compatibility of insecticide and fungus when dual applications are being considered, is clearly of considerable importance. Indeed adverse effects of insecticides and other pesticides on entomopathogenic fungi have often been recorded e.g. DDT and malathion were inhibitory towards *Entomophthora exitialis* pathogenic to the alfalfa aphid (Hall and Dunn, 1958). Therefore it is gratifying that, under the conditions of the experiment, Dimilin had no significant effect on germination and growth of *M. anisopliae* (Table 28). In contrast Dimilin had a slight inhibitory effect on growth of *Verticillium lecanii* (Hall, 1981), no effect on *Beauveria bassiana* and promoted the growth of *Nomuraea rileyi* (Gardner *et al.*, 1979), *in vitro*.

The lack of effect of Dimilin on mycelial growth is perhaps not as important, from a practical point of view, as the absence of inhibition of germination, because *in vivo*, mycelial growth occurs primarily within the host where the pesticide will either be absent or in very low concentrations (Hall, 1981) n.b. the half life of the insecticide in *Schistocerca gregaria* is only 24 h (Ker , 1977).

Using the results of laboratory or *in vitro* experiments to predict the outcome of interactions *in vivo* or in the field may have little value. A pesticide which is deleterious under laboratory conditions may not be in the field e.g. the

fungicide Triarimol (Wilding, 1972) and the insecticide Fenthion (Easwaramoorthy *et al.*, 1978) were toxic towards *Cephalosporium* spp. *in vitro,* but did not hinder the action of the fungus against *Aphis gossypii* and *Coccus viridis* in the field, respectively. However, it seems unlikely that the converse situation would arise, viz. *in vitro* compatibility with *in vivo* antagonism because environmental detoxification (biotic and abiotic) would reduce rather than enhance antifungal properties of an insecticide. The only situation where *in vivo* antagonism might occur is, if a metabolite of the insecticide was fungitoxic.

The time to germination of conidia of *M. anisopliae* on the cuticle of *Manduca sexta* was dramatically reduced when the conidia were presoaked for 20 h in distilled water. Thus 84% of pre-soaked conidia germinated after 24 h, whereas only 37% of fresh conidia germinated in the same time period.This observation is consistent with the results of *in vitro* experiments carried out by Dillon (unpubl.). He found that conidia of the same strain (M.E.I.) germinated in ½ the time if they were pre-soaked in distilled water before suspension in S.D. broth.

McCauley *et al.* (1968) found ca. 100% germination of conidia of *M. anisopliae* on Elaterid cuticle 24 - 48 h post-inoculation. This is considerably more than the 37% of fresh conidia (comparable treatment) that germinated after 24 h on

Manduca cuticle (see above). Clearly a reflection of the different strains of *Metarhizium* used and the peculiarities of the chemistry of *Manduca* and Elaterid cuticles.

The ability of a period of pre-soaking of conidia in water to speed up and synchronise the process of germination when a suitable nutrient source is available does not appear to have been reported before. There is no evidence to support the most obvious conclusion, namely that soaking removed a germination inhibitor. Indeed a culture medium (S.D. broth) previously used to germinate fresh conidia of *M. anisopliae* was not inhibitory to the germination of further batches of fresh conidia (Dillon, unpubl.). However, this does not preclude the possibility that pre-soaking caused either the breakdown of an inhibitor, or the release of a stimulant internally within conidia.

Whatever the reason for the acceleration of germination following pre-soaking of conidia in distilled water, the treatment also resulted in a higher mortality among second instar *Manduca* larvae than when fresh conidia were used. Presoaking enhanced the pathogenicity of *Metarhizium* towards 6 h ± 6 h second instar larvae at both the concentrations of conidia employed (0.1×10^6 and 2×10^6 conidia/ml). The value of the treatment was further in evidence when the period of optimum high humidity (100%) was restricted to 30 h. Not

only was mortality in the fresh treatment reduced but consistenty fewer insects died from mycosis, in contrast , the P treatment was not adversely affected by this regime. However, when a 2 x 15 h period of high humidity were sandwiched either side of a 9h period of relatively low humidity, both F and P failed to realise their full potential and there were no significant differences between the two treatments with respect to either % mortality or % mycosis. This is perhaps not unexpected. Although P was considerably more advanced than F in the rate of germination and appressorial formation, it still took 24 h before ca. 46% of the P germ tubes had formed appressoria.

In the experiments where dual applications of Dimilin and *Metarhizium* were made, pre-soaked conidia were consistently found to produce greater mortality than fresh conidia, supporting the results of experiments described above.

The effect of pre-soaking of conidia on the pathogenicity of *Metarhizium* is very interesting, but from a practical point of view could be nothing more than a curious novelty. Studies such as those of Hall (1982) have shown that the addition of nutrients to a formulation of an entomopathogen enhances the control of a pest population on a crop. This is apparently due to the increase in inoculum potential that occurs from the saprophytic growth of the fungus on leaf surfaces promoted by the formulated nutrients. However, Allen (unpubl.) in our

laboratory has shown that a dearth of nutrients promotes appressorial formation by *M. anisopliae* (M.E.I.). Therefore an alternative approach to the use of such pathogens, where a "quick" kill is necessary, may be a large, pre-soaked inoculum, devoid of added nutrients.

There was a positive correlation between age of the larva (h during the second instar) and the size of the fungal inoculum required for high mortality. The fungus is constrained by the time taken to germinate and then penetrate the cuticle. If ecdysis intervenes prior to penetration then the inoculum may be lost (Zacharuk, 1973; Schabel, 1976; Fargues and Vey, 1977). Therefore the more conidia applied the greater the chance that a fatal level of invasion will occur. However, older larvae will also have a thicker cuticle which will provide a greater barrier to penetration (David, 1967).

In those groups of individuals dosed with *Metarhizium* alone, deaths from mycosis tended to occur prior to or during ecdysis (viz % mycosis was high when % failing to ecdyse was high). Few of the insects that ecdysed successfully subsequently died from mycosis, whether the insects were dosed at 6 h ± 6 h or 30 h ± 6 h. Clearly ecdysis provided an escape from fungal infection in this insect (Zacharuk, 1973) and infections were not carried over the ecdysis.

Most deaths in the combined insecticide/fungus treatments

were due to mycosis, adding support for the hypothesis that synergism was the result of Dimilin facilitating the entry of the fungus into the insect (see Chapter 4).

Dual applications of low toxic doses of Dimilin (ED50) and *Metarhizium* (ED50) acted synergistically against *Manduca*. The level of synergism was at best supplemental, at worst independent, depending on the age of the insect at the time of treatment. Telenga (1959) also achieved supplemental synergism against *Cydia pomonella* using LC 8.8 DDT and LC 10.9 *Beauveria bassiana*. However, most other published studies on dual applications of fungus and toxic doses of insecticide do not achieve better than subadditive synergism (see Table II Benz, 1971).

Future studies on the combined action of Dimilin and *Metarhizium anisopliae* against *Manduca* should attempt to vary the concentrations of the two agents. However, it is unlikely that a sublethal dose of Dimilin would act synergistically with *Metarhizium* because there would be no substantial weakening of the cuticle. Similarly a reduction of the dose of conidia below that giving 26% kill does not result in synergism.

Dual applications of Dimilin and *Metarhizium* (0.1×10^6 conidia/ml) to 6 h ± 6 h old instar larvae under a cycling humidity regime did not act synergistically. In contrast the

identical treatment under constant humidity did result in synergism. However, the susceptibility of 30 h ± 6 h larvae to either F or P conidia was not reduced when the insects were maintained under a cycling humidity regime when compared to that under a constant high humidity. Synergism also occurs under both humidity regimes.

When the two agents were applied simultaneously (rather than separately) they conspicuously failed to act synergistically and mortality in the Dimilin-only treatment was very low. Indeed the latter was conspicuously lower than expected from the results of the contact dose experiment in Chapter 1. However, Dimilin was applied by spraying in Chapter 1, whereas here the insect was dipped in Dimilin suspensions. The performance of the fungus (based on % mortality and % mycosis) was similar in single and combined treatments confirming that Dimilin had no adverse effect on the germination and growth of *Metarhizium*.

CHAPTER 4

THE EFFECTS OF DIFLUBENZURON AND *METARHIZIUM ANISOPLIAE* ON THE FINE STRUCTURE OF THE CUTICLE OF THE 4TH LARVAL INSTAR OF *MANDUCA*

Introduction

It was shown in the last chapter that Dimilin and *Metarhizium anisopliae* combine synergistically to kill larvae of *Manduca sexta*. The hypothesis was made that weakening of the cuticle caused by the Dimilin facilitated the entry of the fungus. Therefore an ultrastructural study was initiated to investigate this possibility.

Most studies on the effects of Dimilin on the structure of insect cuticle have involved light microscope histochemistry, e.g. *Pieris brassicae* (Gijswijt *et al.*, 1979); *Leptinotarsa decemlineata* (Grosscurt, 1978); *Boarmia bistortata* (Salama *et al.*, 1976). The only electron microscope study is that of Ker (1978) on *Schistocerca gregaria*.

In some insects no stable cuticle is deposited during Dimilin treatment (Mulder and Gijswijt, 1973). Post-ecdysial Dimilin-affected cuticle of *Pieris brassicae* for example consisted solely of dense globules, possibly of proteins which stained red with Mallory's triple stain (Gijswijt *et al.*, 1979). By comparison cuticle from the proximal end of the hind tibia of the adult locust, *Schistocerca gregaria*, was

stabilised sufficiently under Dimilin treatment to produce a discrete layer (Ker, 1977).

There are a number of fine structural studies on the penetration of insect cuticle by entomopathogenic fungi (e.g. *Metarhizium anisopliae*/Elaterid larvae (Zacharuk, 1970); *Entomophthora apiculata/Trichoplusia ni* (Lambiase and Yendol, 1977); *Nomuraea rileyi/Heliothis zea* (Mohamed *et al.*, 1978); *Nomuraea rileyi/Anticarsia gemmatalis* (Boucias and Pendland, 1982)).

The most comprehensive and influential of these is that of Zacharuk on the interaction between *Metarhizium anisopliae* and wireworm larvae (Zacharuk, 1970 a,b,c; 1971; 1973; 1974). However, Zacharuk's work is the only electron microsope study on the histopathology of the green muscardine in insects.

In the light of the above it was hoped that the work presented in this chapter would not only shed some light on the synergism between Dimilin and *M. anisopliae* but add to our knowledge on the effects of the single treatments as well.

Materials and Methods

The insects employed were newly moulted (ca. 0 h) fourth instar larvae that had been reared on artificial diet since hatching (see General Materials and Methods). Three insects were used in each of four treatments:

1. Insects fed Dimilin
2. Insects treated topically with conidia of *Metarhizium*
3. Insects treated topically with conidia and fed Dimilin
4. Control insects

The insecticide was applied to the food by dipping blocks of artificial diet in 0.02% aqueous suspension of Dimilin (ED80 dose, see Chapter 1) as described in Materials and Methods Section to Chapter 1. Topical application of conidia was achieved by placing a 5 µl aliquot of 1 x 10^8 conidia/ml on the tergite of the third abdominal segment using a microcap. The conidial suspension was made up in 0.4% Tween 80 as described in the Materials and Methods section to Chapter 2.

Insects of all treatments were fed diet minus the antifungal compounds formalin, sorbic acid and methyl-p-hydroxybenzoate (see Chapter 2) for 48 h, then complete diet for the remaining period of the experiment. The insects were kept individually in small Petri-dishes (50 mm diameter) and stored in plastic boxes (see Materials and Methods, Chapter 2) lined with water-soaked cotton wool to provide a high humidity within an environmental cabinet (Fisons) under a temperature of 25°C and 17 h light : 7 h dark photoperiod.

One insect was sacrificed per treatment at each of 24, 48 and 72 h after the start of the experiment. An additional insect was killed ~~immeidately~~ immediately after ecdysis to the fourth instar. Death was caused by decapitation and the tergite of the third abdominal segment excised and cut into 4 - 5 pieces using a sharp knife.

The integument was processed using the tannic acid method described by Locke and Huie (1980). The tissue was fixed in a solution of 2.5% glut~~e~~araldehyde, 5% tannic acid (neutralized with NaOH) and 2% sucrose in 0.05 M sodium phosphate buffer pH 7.2 for 5 - 8 h at room temperature. The tissue was then washed three times in 4% sucrose in 0.05 M sodium phosphate buffer pH 7.2, 15 minutes each wash, at room temperature. At this stage the material was left overnight in a fresh aliquot of the washing medium at 4°C. Post fixation was carried out in 0.05 M sodium phosphate buffer, pH 7.2 containing 1% osmium tetr~~a~~oxide, and 4% sucrose, for five hours. The osmium was then removed by washing with buffer as described above and left overnight in a fresh aliquot of buffer at 4°C.

Dehydration of the material was effected by passage through an acetone series; three changes of 10 - 15 minutes in each of 30%, 50% and 70% acetone. The material was then left for 12 h in 70% acetone at 4°C prior to a final wash of 3 changes, each of 10 - 15 minutes, in 100% acetone at room temperature.

The material was transferred to a mixture of 100% acetone

and TAAB standard epoxy resin (see below) 1:1 (v/v) for 24 h at room temperature. The acetone diluted resin was then replaced with two changes of neat resin (24 h and 12 h respectively). Finally the material was placed in fresh resin and polymerisation was effected at 70°C for sixty hours.

Silver/silver-gold sections were cut on a Reichert OMU3 ultramicrotome fitted with a diamond knife, expanded with diethyl ether vapour and mounted on 200 mesh copper grids coated with either pioloform or formvar. Sections were stained with uranyl acetate and lead citrate (10 minutes each) (Reynolds, 1963) prior to their examination in a JEOL 100 CX transmission electron microscope.

Results

Ultrastructure of the cuticle of fourth instar larvae

The cuticle of the newly moulted (zero hour old) fourth instar larva is characterized by the presence of prominant projections or papillae (Figs 1 and 2). There is a certain amount of dense material between the papillae which may be vestiges of the moulting fluid. The procuticle consists primarily of 10 - 12 lamellae, amorphous cuticle and pore canals.

During the succeeding 48 h of the instar the papillae became less prominent as the epicuticle unfolds, and the procuticle increases in thickness ca. x 4 (Figs. 3 and 4). The lamellae also increase in number ca X5 and the apical ones (the first to be laid down) become thinner due to deformation during the growth of the larva. Finally between 48 - 72 h a new cuticle is formed and much of the old broken down (Fig. 5).

Epicuticle

Two layers are readily distinguished; a thin electron dense layer which corresponds to the "cuticulin" of Locke (1966) and the "outer epicuticle" of Weis-Fogh (1970); and a thicker, lighter layer which is similar to the "protein epicuticle" of Locke (1969) and the "inner cuticle" of Weis-Fogh (1970) (Figs. 1, 6, 7 and 10). Locke's (1966) terminology will be adopted in the present work.

A certain amount of "fluffy" material is present on the

surface of the epicuticle. This may be a remnant of the wax and cement layers (Weis-Fogh, 1970) which have largely been removed during fixation of the material (Fig. 6). Wax canals ramify through the inner epicuticle and open at the surface of the outer epicuticle (Figs. 6 and 10).

Procuticle

The procuticle forms the bulk of the cuticle. The region immediately below the epicuticle is amorphous viz. there is no ordered arrangement of fibrils (Figs. 1, 2, and 7). During the course of the instar dense vesicles, apparent in the amorphous cuticle of newly moulted insects (Fig. 1), increase in number (Fig. 7). These may be deposits of melanin (Locke, 1966). Wax canal filaments are also very prominent (Figs, 6, 9, 10).

The helicoidal fibrils (Neville, 1975) which make up the lamellae of the procuticle (Figs. 1, 2, and 3) may be contrasted with the fibrils of the pore canals, which are laid down perpendicular to the plane of the epidermis (Figs. 1, 2, and 3). Pore canals with associated electron dense filaments are consistent features of this region of the procuticle (Fig. 9).

Subcuticle

The granular dense zone immediately below the lamellated procuticle and above the epidermis, the subcuticle (Figs. 1,

3 and 4) is believed to be the site of orientation of cuticular fibrils (Neville, 1975).

Epidermis

The apical surface of the epidermis cells is microvillated. In the regions of lamellated cuticle the microvilli are short and very regular in appearance (Figs. 14 and 17). However, a group of very long thin microvilli are present forming each pore canal (Fig. 9). The electron dense membrane plaques believed to be the site of synthesis of the cuticle (Locke, 1976) are present on the apical surface of small microvilli (Figs. 14 and 17).

The effect of Dimilin on the fine structure of the cuticle of fourth instar *Manduca* larvae

The cuticle of larvae treated with Dimilin for the first 24 and 48 h of the fourth instar presents a very different profile from that of normal cuticle. Although the outer third of the procuticle is lamellated normally, the rest of the procuticle lacks organisation and there is no subcuticle (Figs. 3, 4, 19 and 20). The only features of this otherwise bland granular zone are electron dense globules and long microvilli from the epidermis (Figs. 8 and 19). Despite the malformation of Dimilin-treated cuticle, its overall thickness (24 μm) is similar to that of cuticle from normal 24 h old

insects (23 µm; Figs. 3 and 19). However, the affected cuticle may be deposited irregularly because the inner surface of the cuticle, and hence the plasma membrane of the epidermal cells, is convoluted (Figs. 14-17, 19). The shape and number of lamellae in the apical regions of the cuticle is similar to that in the cuticle of newly moulted animals (Figs. 1, 11, 19 and 20). Therefore this area of cuticle was clearly laid down pre-ecdysially before treatment with Dimilin was started.

The site of origin of the aberrant procuticle, the plasma membrane microvilli of the epidermis, seem equally disturbed by the Dimilin. The microvilli do not have a regular outline and membrane plaques are neither a consistent feature nor, when present, as electron dense as those in the normal insect. This is true for both standard microvilli (Figs. 14, 16 and 17) and long microvilli that form the pore canal filaments (Figs. 8 and 9). In addition the small microvilli are out of contact with the newly deposited cuticle (Fig. 16). By 48 h the epidermal cells of Dimilin-treated animals have many large cytoplasmic extrusions on their apical membranes (Figs. 15 and 20). The Dimilin affected cuticle is still degraded normally 48 - 72 h after the ecdysis to fourth larval instar (Figs. 12 and 13).

The new fifth instar cuticle laid down 48 - 72 h after ecdysis to fourth instar (Fig. 8) exhibits a similar appearance to that of the Dimilin-treated intermoult cuticle (Figs. 19 and

20). Once again the featureless Dimilin-affected new cuticle is in stark contrast to the lamellated normal new cuticle (Figs. 8 and 9).

The fine structure of *Metarhizium anisopliae* and its penetration of the cuticle of fourth instar *Manduca* larvae

The fine structure of germinating conidia of *Metarhizium* revealed by the present work is similar to that described by Zacharuk (1970a). Fig. 22 shows a conidium that is in the final stages of swelling prior to germ tube formation. Characteristically, the lipid inclusion shows granulation of its contents, the plasmalemma is separating from the wall and the oil droplets are becoming vacuolated.

A germ-tube finally breaks through the trilaminate conidial wall, surrounded by its own primary germ-tube wall (Fig. 23). The conidium left behind is now very much less electron dense. The contents of the lipid inclusion have almost completely disappeared leaving a large vacuole and there is more endoplasm and less space taken up by cellular organelles. Mitochondria congregate in the vicinity of the germinating pole of the conidium and are the first organelles to enter the germ-tube.

An appressorium is usually formed at the point of contact of the germ-tube with the cuticle (Fig. 25)and a septum is

formed between the germ-tube and conidium as in Fig. 24 where the germ-tube is very short and the septum is close to the conidium. The appressorium may proliferate to form additional ones (Figs. 25 and 35) which may be "welded" together by mucous-like material to form an infection cushion (Zacharuk, 1970b). The appressoria in Fig. 25 have clear primary and secondary walls and the absence of empty conidia in this infection cushion denotes the formation of the appressoria by long germ-tubes. The appressorium may vary in size and shape depending on the plane of section (Figs. 24, 25 and 35). However, observations of cuticular replicas made with the light microscope (Chapter 3, Fig. 1) suggest that the majority of appressoria are clavate in form (see Fig. 24 of present chapter). Schabel (1978) made a similar observation during the course of a scanning electron microscope study of *Metarhizium anisopliae* on *Hylobius pales*.

Unfortunately, the initial stages of cuticular penetration were not observed with normal insects. However, in Dimilin-treated insects infection plates were seen penetrating the epicuticle 24 h after inoculation (Fig. 26). A similar time scale may operate within the non-treated insect, because in both treatments penetrant hyphae are found throughout the cuticle (Figs. 27 and 35) and hyphal bodies are present within the epidermis 48 h post-inoculation (Fig. 30). Growth of penetrant hyphae occurs laterally between the lamellae of the procuticle. Deformation of the lamellae surrounding the

hyphae indicates that penetration is primarily mechanical (Figs. 27 and 28). However, the clearing of the procuticle in the vicinity of the hyphae suggests there may also be a histological component to the process as well (Figs. 26 and 28).

Vertical growth of penetrant hyphae, essential if invasion of the haemocoel is to take place, occurs mainly down pore canals (Figs. 29, and 36). Presumably these are pathways of least resistance, as growth is with rather than against the "grain" of the cuticle.

Sections of penetrant hyphae are regular in outline, they are surrounded by an electron transparent primary wall (Figs. 27 and 28), which is similar to the primary wall of the appressorium (Fig. 24) and the electron transparent wall of the penetration plate (Fig. 26). Lateral penetrant hyphae are non-electron dense and granular in appearance (Figs. 27 and 28). Many small mitochondria, elements of rough endoplasmic reticulum and lipid inclusions are present, indicative of high metabolic activity (Zacharuk, 1970b). Vertical penetrant hyphae are considerably more vacuolated but still have many osmiophilic/lipid droplets (Figs. 29). Lateral and vertical penetrant hyphae were essentially similar, in contrast to the type 1 and type 2 hyphal bodies of Zacharuk (1970b).

Fine structural study of the penetration of Dimilin-affected cuticle of fourth instar *Manduca* larvae by *Metarhizium anisopliae*

The initial stages of penetration were observed in Dimilin-treated insects 24 h post inoculation (Fig. 26). Appressoria produce short penetration pegs which traverse the epicuticle, causing only minimal mechanical disruption (Figs. 25 and 35). The peg expands to form a penetration plate immediately below the epicuticle within the amorphous procuticle (Figs. 25, 26 and 35). A thin penetrant hyphae is produced by the penetration plate usually at its lateral edge. This grows obliquely towards the lamellated procuticle and the area in advance of the penetrant hyphae appears histolysed (Fig. 26). On reaching the lamellate procuticle the penetrant hyphae extends laterally between the lamellae. Fourty-eight hours post-inoculation, hyphae have penetrated through the cuticle into the epidermis. This is a similar time scale to that achieved by the fungus on normal cuticle (Fig. 30). However, unlike normal cuticle (Figs 27 and 29), cuticle in the Dimilin-treated insect provides little mechanical resistance to penetrant hyphae, and falls apart during invasion (Fig. 35).

The mode of penetration of *Metarhizium* through Dimilin-affected cuticle is similar to that through normal cuticle. Penetrant hyphae are seen growing laterally between lamellae in the pre-ecdysial cuticle, while vertical penetration occurs through pore-canals (Figs. 35 and 36).

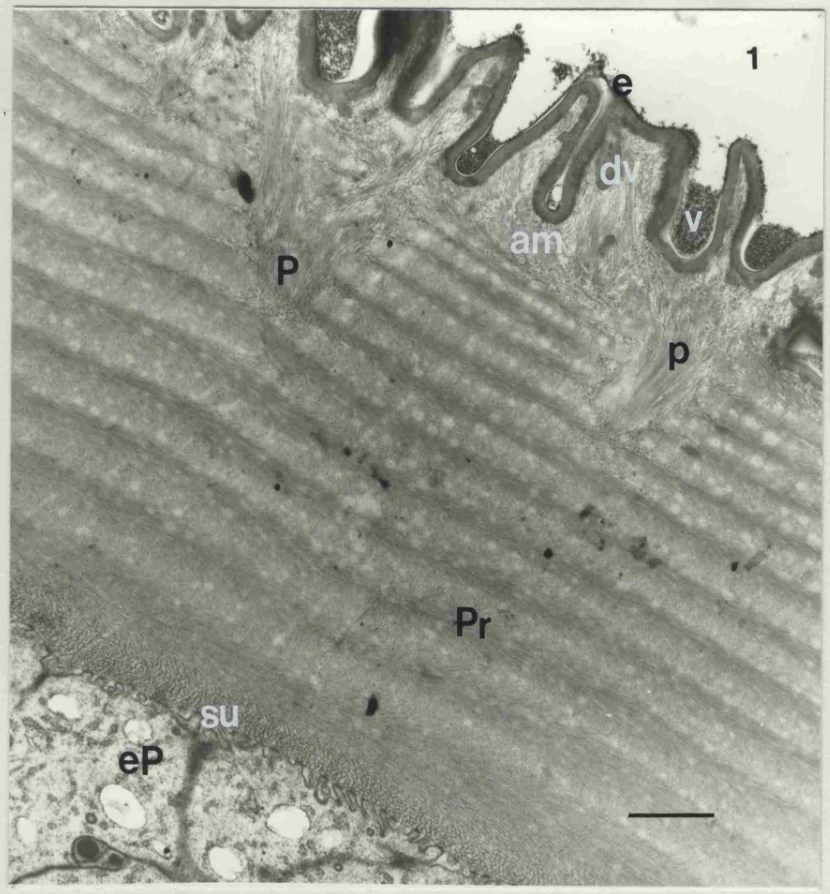
Once a vertical penetrant hypha has passed down a pore canal through the lamellated pre-ecdysial cuticle, the hyphal tip expands within the Dimilin-disrupted cuticle (Figs. 35 and 36). Growth through the last named is not necessarily continuous and may proceed by the budding of hyphal bodies; particularly during the latter stages of the disintegration of the cuticle. (Figs. 32 and 35). The expansion of the penetrant hypha within the Dimilin-affected cuticle further supports the hypothesis that this area of the cuticle provides little mechanical resistance to the fungus. Indeed the Dimilin-affected intermoult cuticle is so completely disrupted by the fungus that it affords no barrier to host haemocytes attracted by the presence of the fungus (Figs. 33, 34 and 35). The thickness of the cuticle 48 h post-ecdysis (19 µm) is considerably less than that in either Dimilin-only treated insects (41 µm), or normal (34 µm), but is comparable to that in conidia-only treated insects (24 µm) at the same stage (Figs. 4, 20, 27 and 31).

Penetrant hyphae within the Dimilin-affected cuticle became surrounded by electron-dense melanin-like material (Figs. 32, 36 and 37). This may originate at least in part from the deposits of electron dense material normally present in Dimilin-affected cuticle (Figs. 19 and 31). However, a contribution may also come from the haemocytes, some of which contain a similar material (Figs. 32 and 33). The deposition of melanin within the cuticle is a common response to

invasion by entomopathogenic fungi (Charnley, 1983). However, in this instance, as in many others, it is an inadequate defence against invasion by *Metarhizium*.

Fig. 1. Low power electron micrograph of the integument immediately after ecdysis to the fourth larval instar. Note that the epicuticle (e) is thrown into folds forming papillae, amorphous region (am), pore canals (p),dense vesicle (dv), vestiges of the moulting fluid (v), subcuticle (su), lamellated procuticle (pr) and epidermis (ep). Bar = 1 μm.

Fig. 2. Apical region of the cuticle of a newly moulted fourth instar larvae. It shows epicuticle (e) amorphous region (am), pore canals (p) and pore canals filaments (pcf), dense vesicle (dv). Bar = 1 μm.

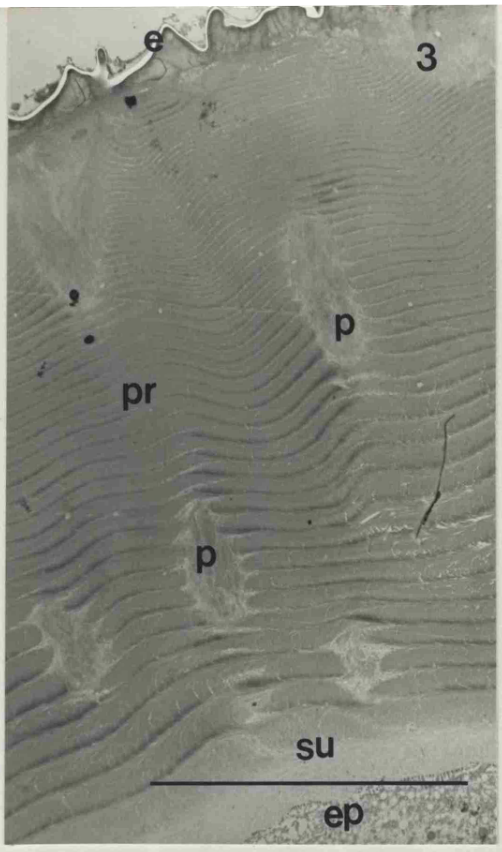

1
e
dv
v
am
P
p
Pr
su
eP


2
dv
am
e
P
Pcf

Fig. 3. An electron micrograph of the fourth larval instar 24 hours after ecdysis and fed on normal food. It shows the epicuticle (e), pore canals (p), lamellated procuticle (pr), subcuticle (su) and epidermis (ep). Bar = 10 µm.

Fig. 4. An electron micrograph of the fourth larval instar, 48 h after ecdysis and fed normal food. It shows epicuticle (e), lamellated procuticle (pr), subcuticle (su) and epidermis (ep). Bar = 10 µm.

Fig. 5. An electron micrograph of a fourth larval instar cuticle 72 hours after ecdysis. It shows the remnants of the old fourth instar cuticle following digestion and absorption, the lamellated exuvia (ex), ecdysial membrane (em), ecdysial space (es), and the newly formed cuticle (nc).

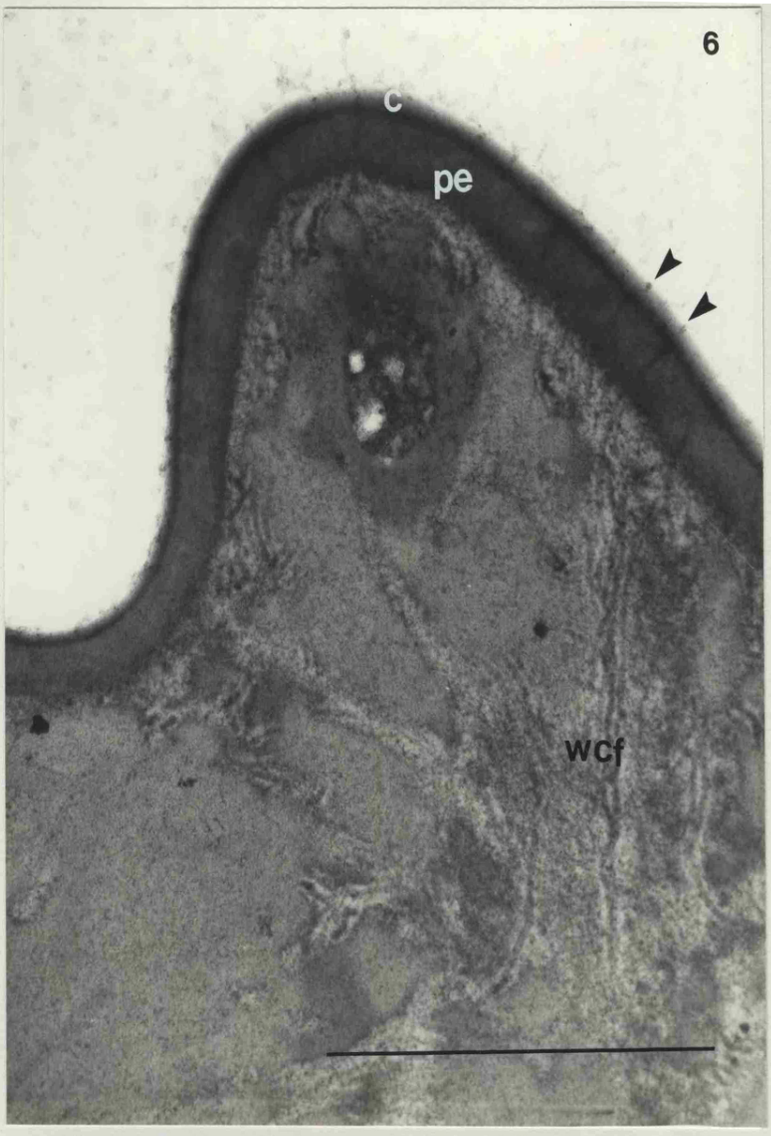

e
3
p
pr
p
su
ep


e
5
ex
em
es
nc


e
4
pr
su
ep

Fig. 6. A high power electron micrograph showing a papilla in cuticle 24 h after ecdysis. The insect was fed on normal food. The micrograph shows the cuticulin (c), protein epicuticle (pe), and wax canal filaments (wcf). Note the tips of ~~(wcf)~~ wcf at arrow heads. Bar = 1 μm.

Fig. 7. Apical region of the cuticle 24 h after ecdysis. The insect was fed on normal food. It shows epicuticle (e), dense vesicles (dv) and amorphous region (am). Bar = 1 μm.


6
c
pe
wcf

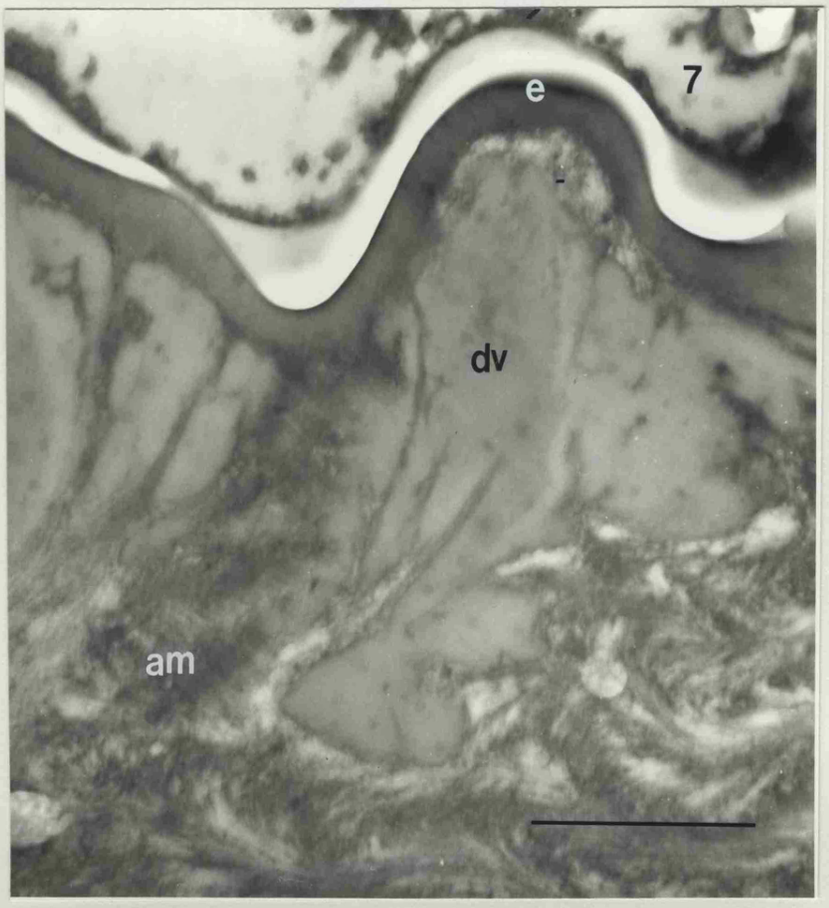

e
7
dv
am

Fig. 8. An electron micrograph showing a newly formed cuticle, 72 h after ecdysis to fourth larval instar in an insect treated with Dimilin. It shows the epicuticle (e), pore canal filaments (pcf) and epidermis (ep). Note the non-lamellated procuticle (de). Bar = 1 µm.
me = melanin-like material

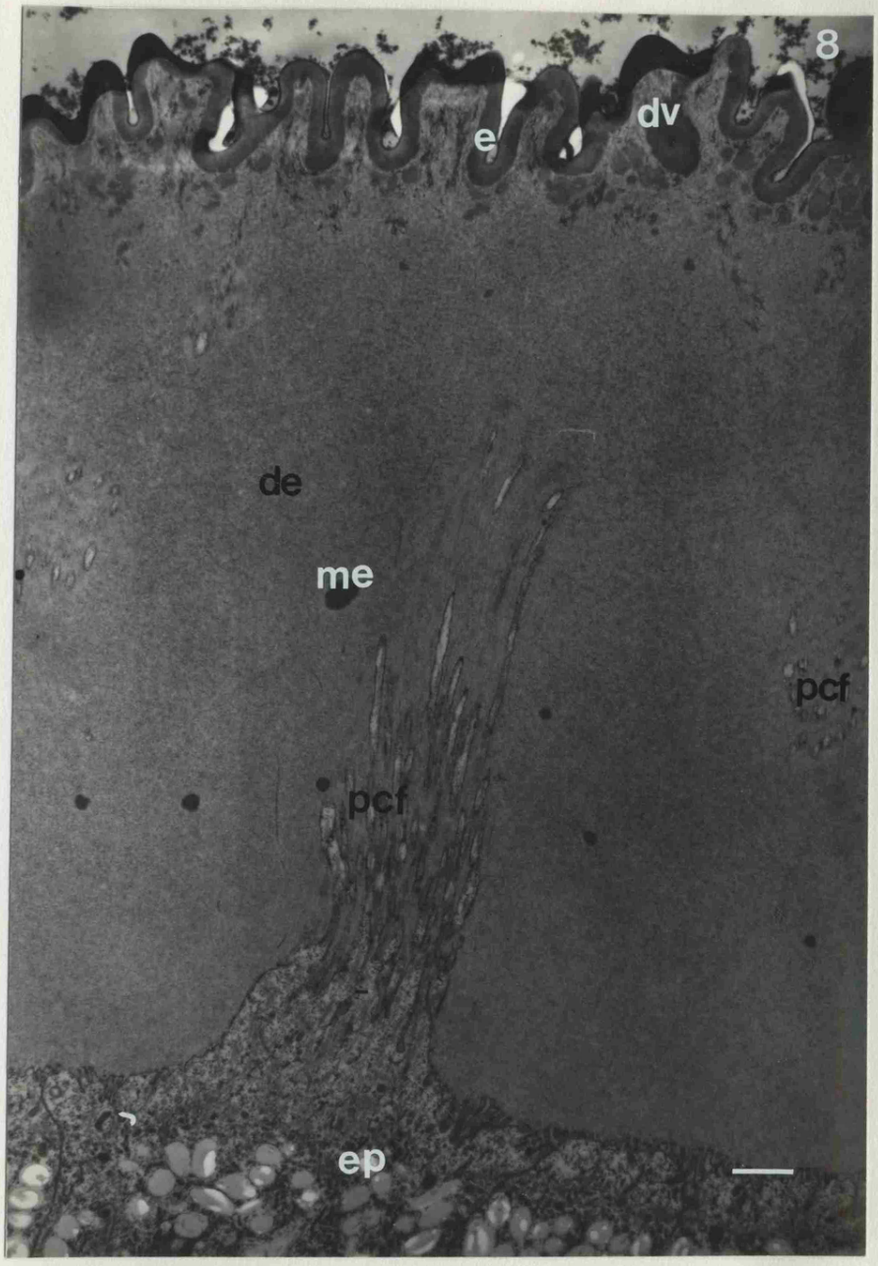

8
e
dv
de
me
pcf
pcf
ep

Fig. 9. An electron micrograph showing a newly formed cuticle, 72 h after ecdysis to fourth larval instar in an insect fed on normal food. It shows the epicuticle (e), wax canal filaments (wcf), pore canal filaments (pcf) and the epidermis (ep). Note the lamellated procuticle, (lm). Bar = 1 µm.

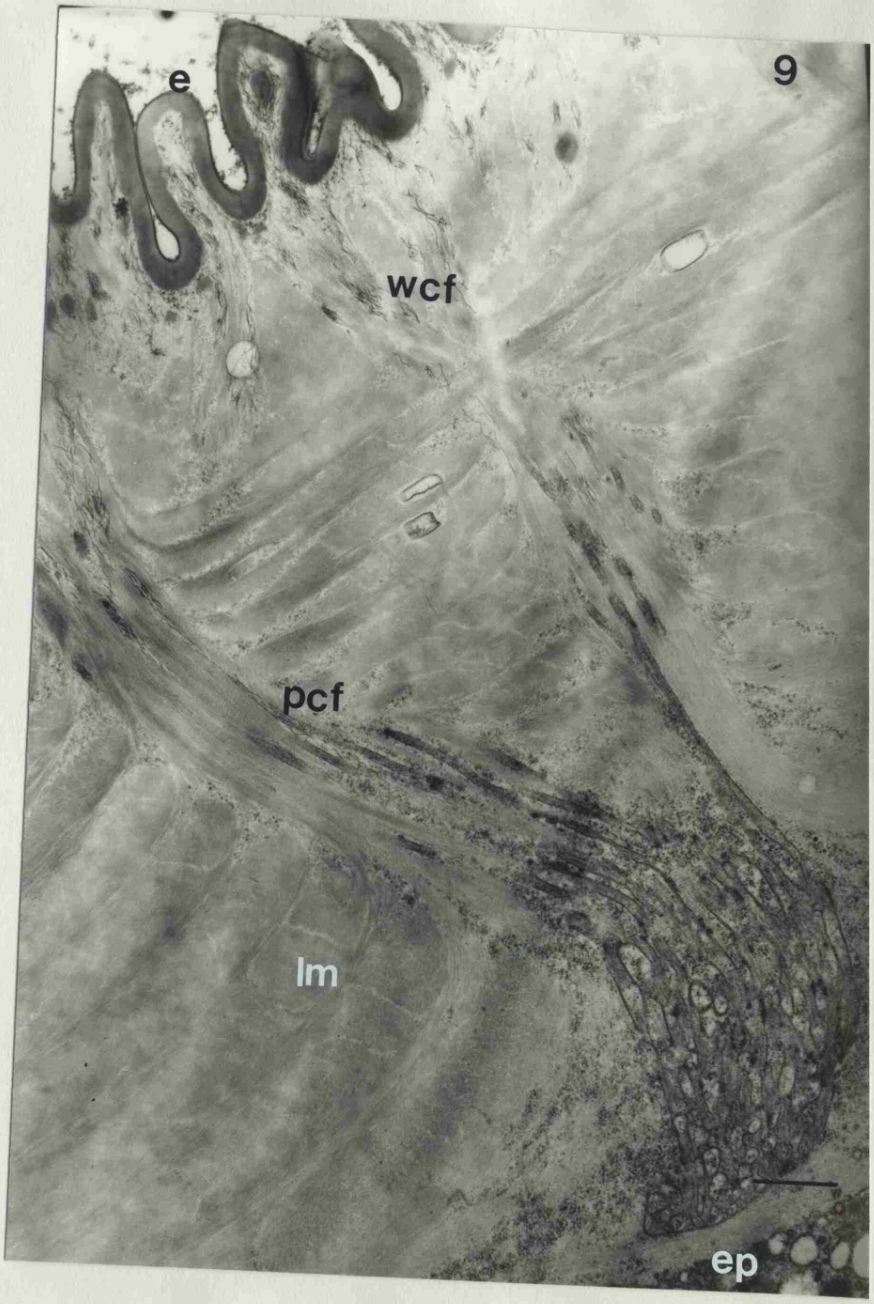
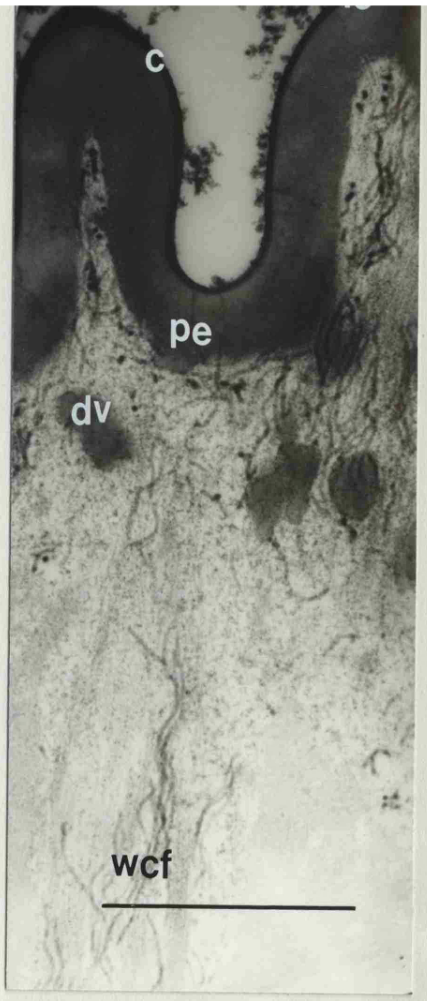

e
9
wcf
pcf
lm
ep

Fig. 10. Apical region of a newly formed cuticle 72 h after ecdysis to fourth larval instar from insect fed on normal food. It shows the cuticulin (c), protein epicuticle (pc) and wax canal filament (wcf) dense vesicle (dv). Note the (wcf) traversing the (pe). Bar = 1 μm.

Fig. 11. An electron micrograph of a fourth larval instar, 48 h after ecdysis, from a Dimilin-treated insect. It shows an organised cuticle (1 m) and a disorganised cuticle (de). Bar = 1 μm.

Fig. 12. An electron micrograph showing the remnants of the fourth instar cuticle following digestion and absorption (exuvia), 72 h after ecdysis to fourth larval instar on a normal insect. It shows the epicuticle (e), lamellated exuvia (ex) and ecdysial membrane (em). Bar = 1 μm.


c
pe
dv
wcf


ex
em

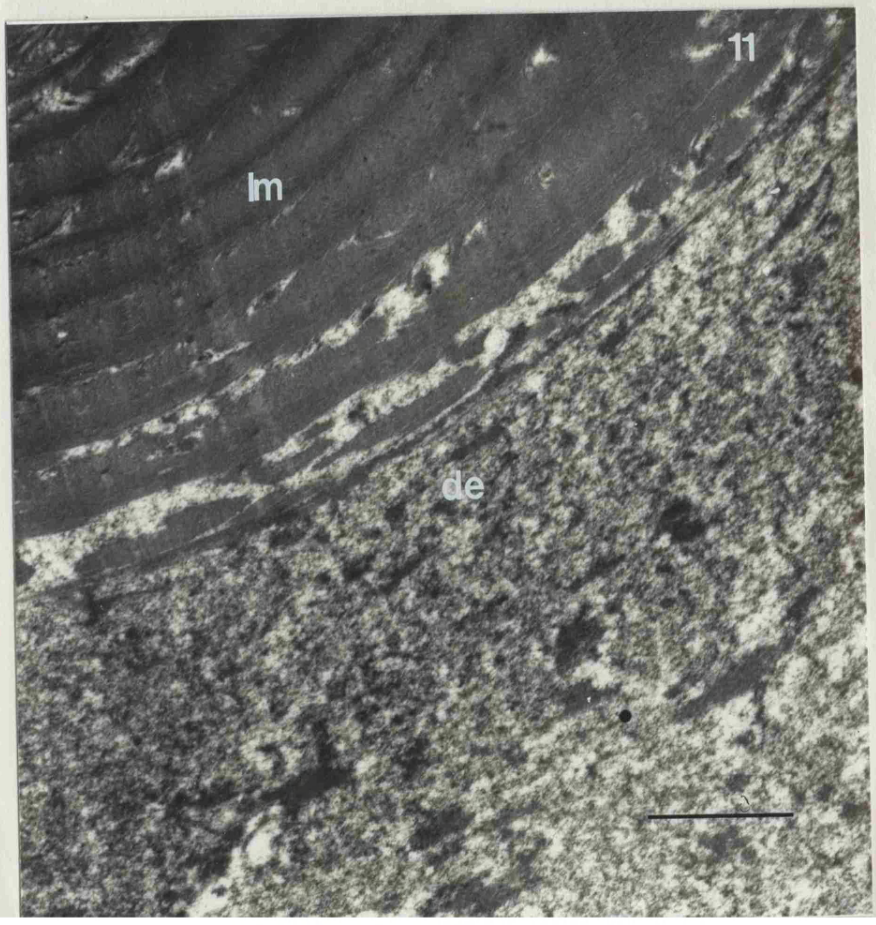
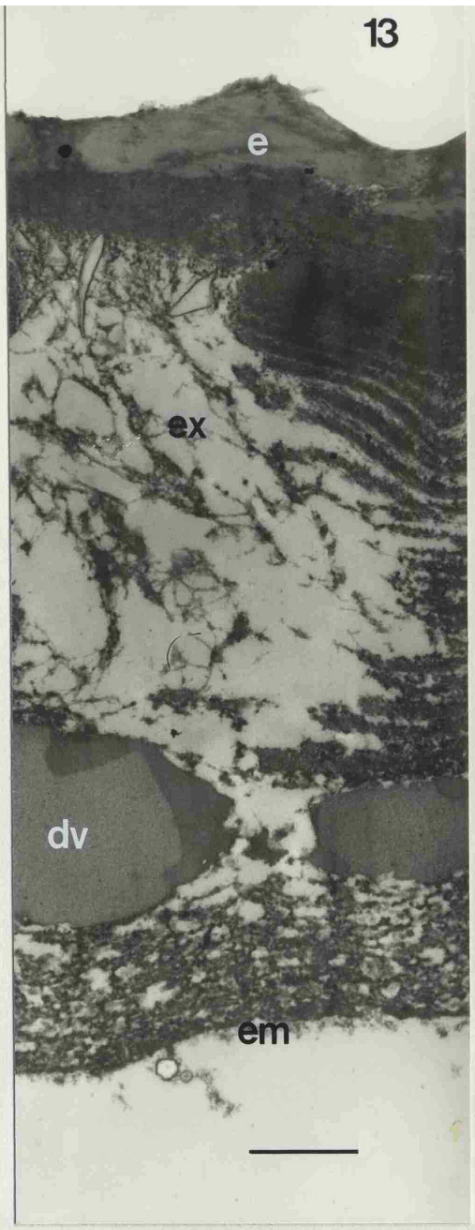

11
lm
de

Fig. 13. An electron micrograph showing the remnants of the fourth instar cuticle following digestion and absorption (exuvia), 72 h after ecdysis to fourth larval instar on a Dimilin-treated insect. It shows the epicuticle (e), lamellated exuvium (ex), and ecdysial membrane (em). Note dense vesicles (dv) appear resistant to digestion. Bar = 1 μm.

Fig. 14. Basal region of the cuticle of a newly moulted fourth instar larva. It shows the lamellae (lm), subcuticle (su) and epidermis (ep). Note the regularity of the microvilli and the line formed by electron-dense plaques on top (arrow heads). Bar = 1μm.


13
e
ex
dv
em

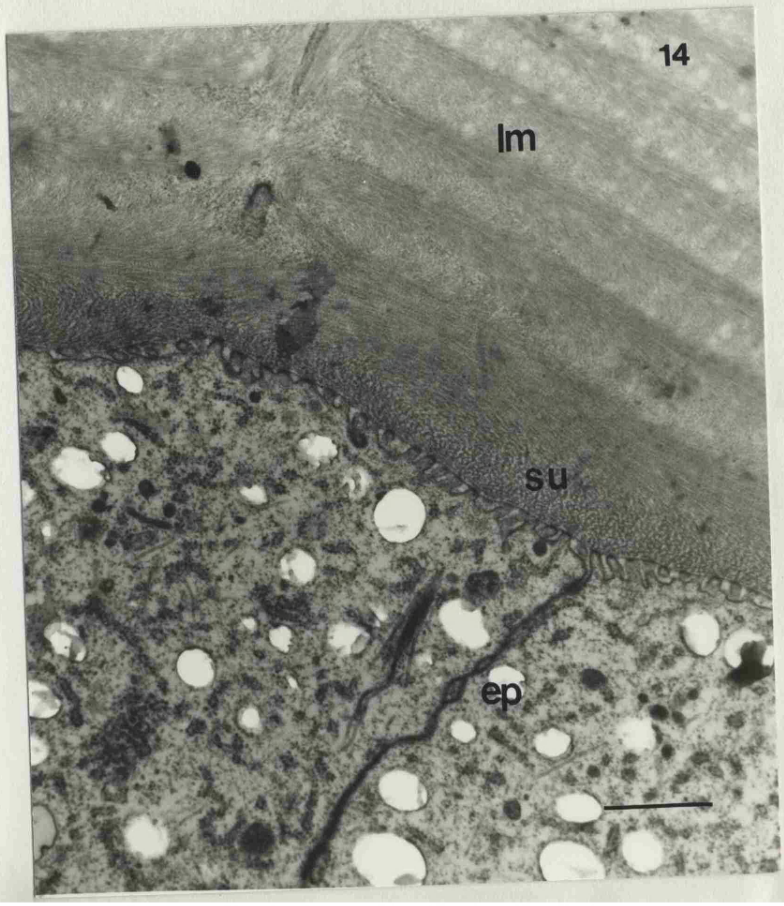
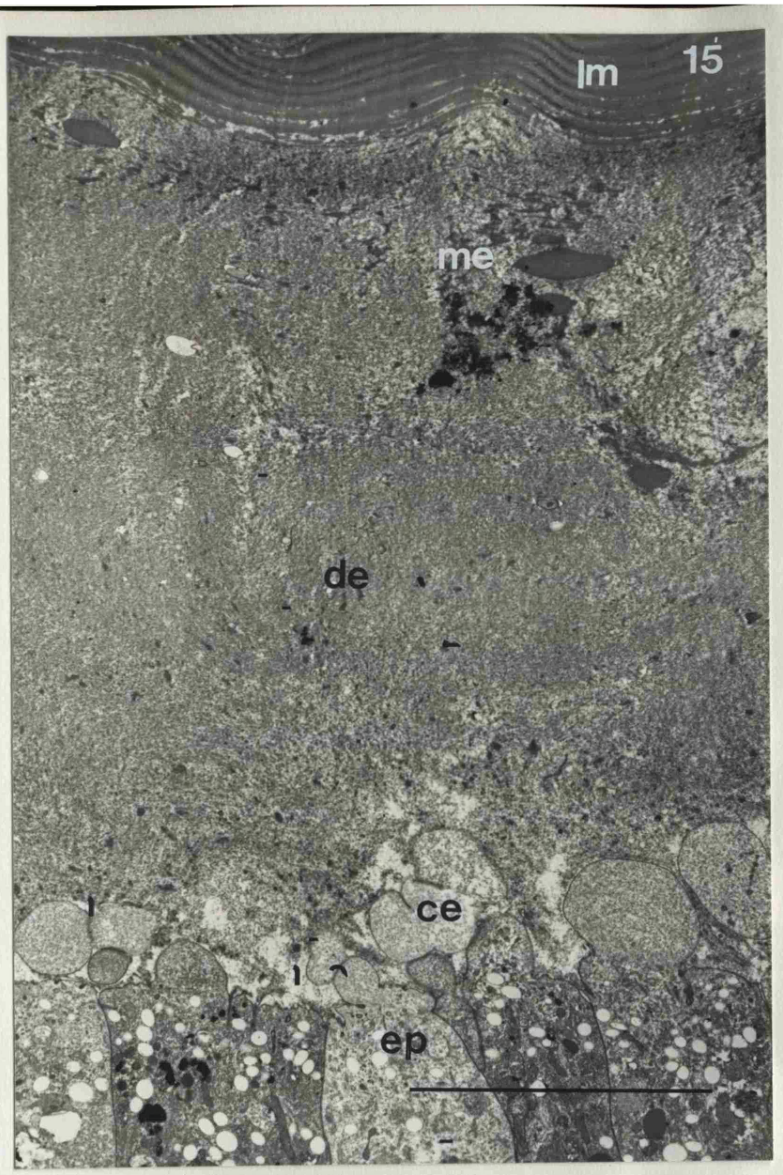

14
lm
su
ep

Fig. 15. An electron micrograph of the fourth larval instar, 48 h after ecdysis and fed on Dimilin. It shows the organised cuticle (lm), disorganised cuticle (de) and epidermis (ep) melanin (me). Note the cytoplasmic extrusions of epidermal cells (ce). Bar = 10 µm.

Fig. 16. Basal region of a cuticle, 24 h after ecdysis to fourth larval instar fed on Dimilin. It shows the disorganised cuticle (de) and epidermis (ep). Note the irregularity of the microvilli and the reduction in electron-density of plaques on top (at arrow heads). Bar = 1 µm.


lm
15
me
de
ce
ep

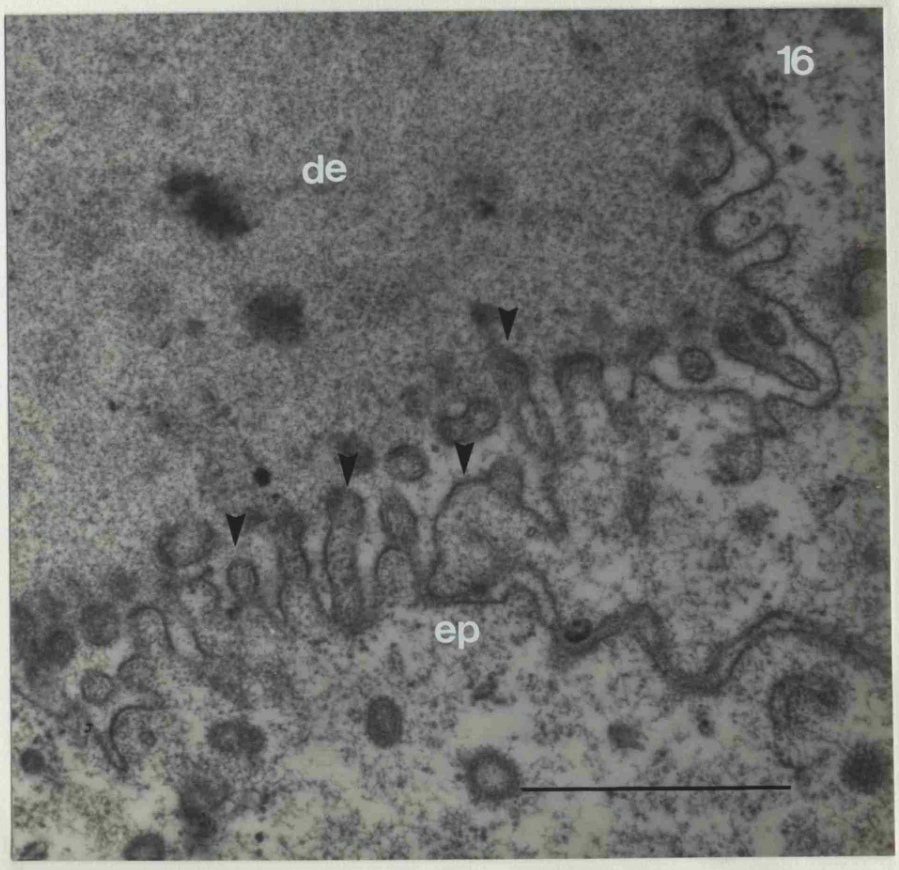
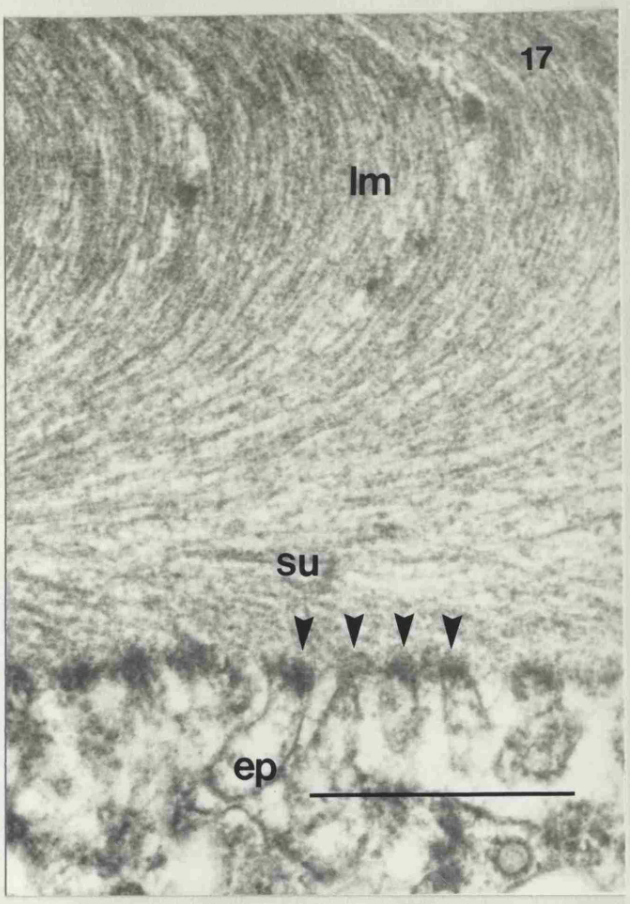

16
de
ep

Fig. 17. Basal region of the cuticle 24 h after ecdysis to fourth instar larva, insect fed on normal food. It shows the subcuticle (su) and epidermis (ep) lamella (lm). Note the regularity of the microvilli and electron-dense plaques on top (at arrow heads). Bar = 1 µm.

Fig.18 An electron micrograph of a fourth larval instar, 48 h after ecdysis, from a Dimilin-treated insect. It shows an organised cuticle (lm), a disorganised one (de) and a melanin-like bodies (me). Bar = 1 µm.


17
lm
su
ep

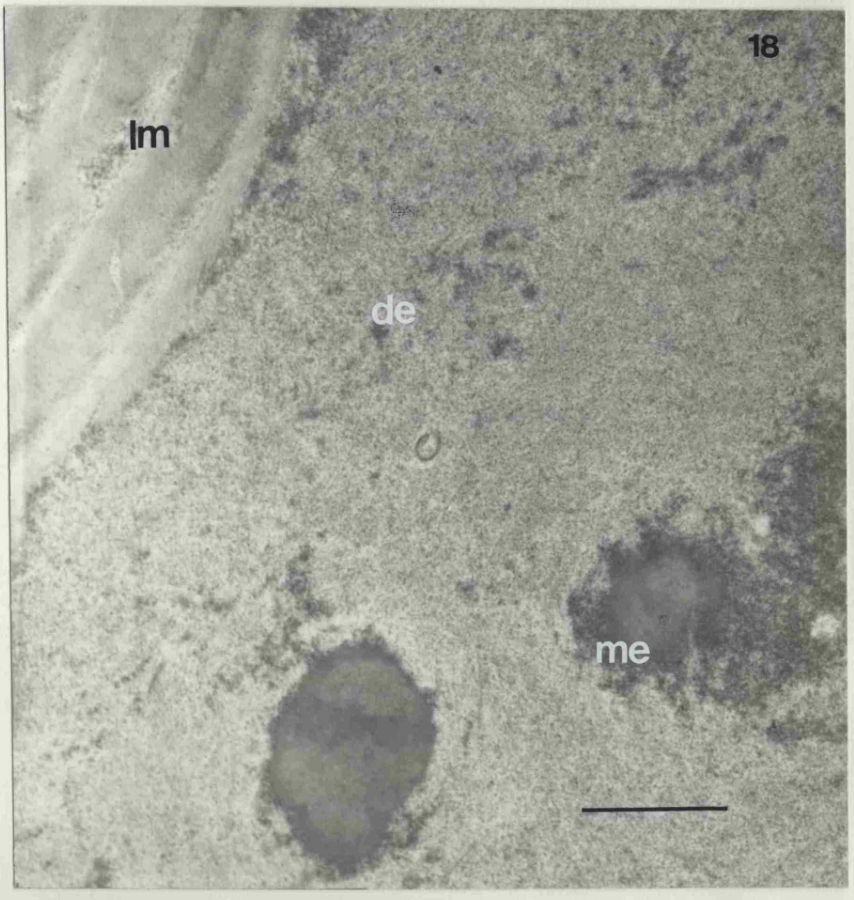

18
lm
de
me

Fig. 19 An electron micrograph of cuticle of a fourth instar larva, 24 h after ecdysis and fed on Dimilin. It shows the epicuticle (e), organised cuticle (lm), disorganised one (de) and epidermis (ep) pore canal filaments (pcf), and melanin (me). Note the undulation of epidermal cells. Bar = 10 µm.

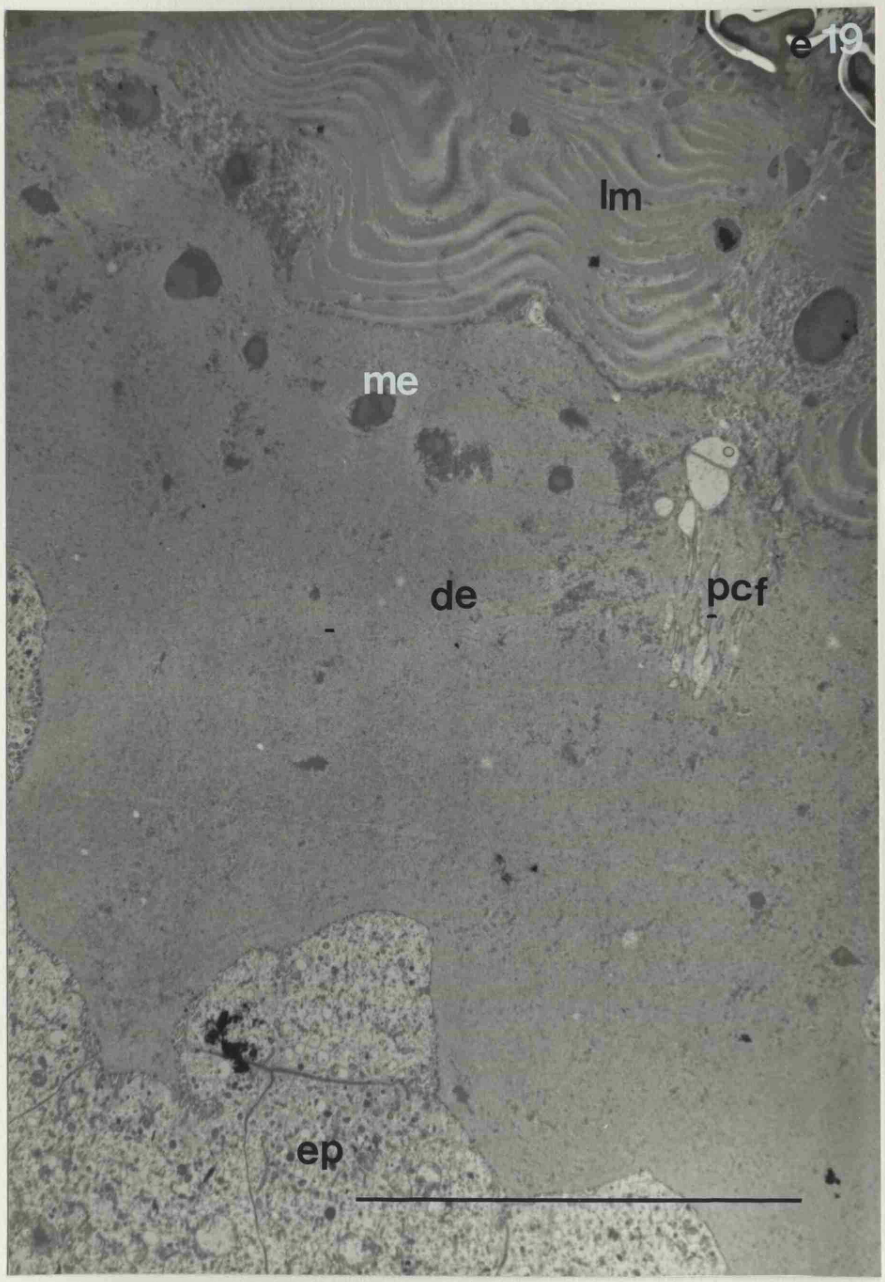
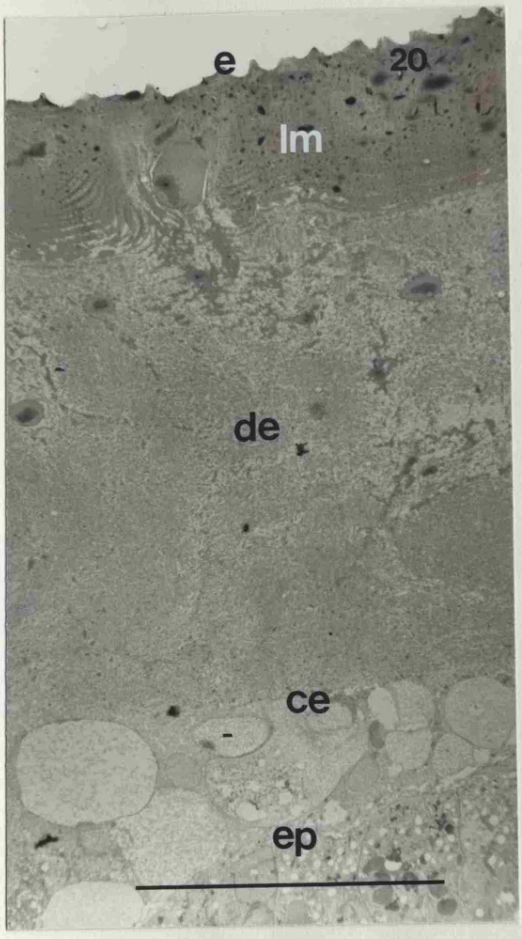

e-19
lm
me
de
pcf
ep

Fig. 20. An electron micrograph of cuticle of a fourth instar larva, 48 h after ecdysis and fed on Dimilin. It shows the epicuticle (e), organised cuticle (lm), disorganised cuticle (de) and epidermis (ep). Note the cytoplasmic extrusions of epidermal cells (ce). Bar = 10 µm.

Fig. 21. An electron micrograph of cuticle of a fourth instar larva, 24 h after ecdysis and fed on Dimilin. It shows the epicuticle (e), disorganised pore canal cuticle (dp) and melanin-like bodies (me). Note the extent of the disorganisation of the pore canal cuticle deep within the organised cuticle (lm). Bar = 1 µm.


e
20
lm
de
ce
ep

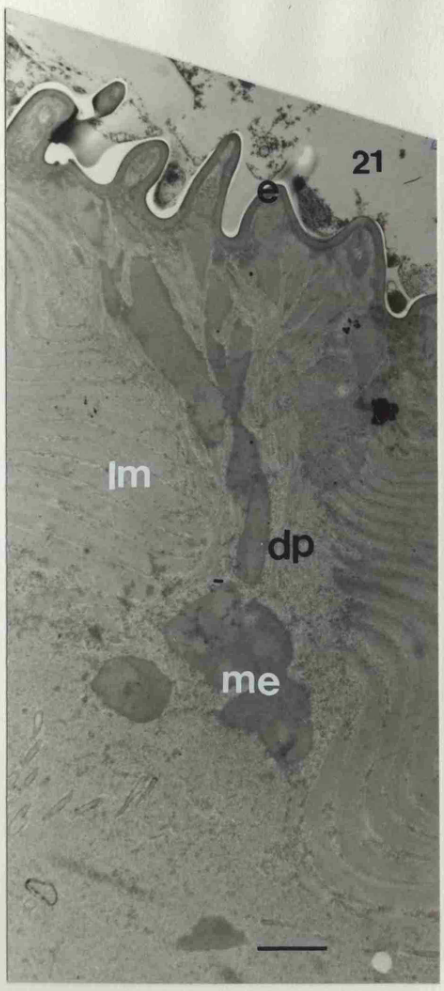

21
e
lm
dp
me

Fig. 22. An electron micrograph of cuticle of a fourth instar larva, 24 h after ecdysis, fed on normal food and treated with conidia. It shows conidial wall protrusion (pt), oil globules (o), lipid inclusion (l) and insect epicuticle (e).
Bar = 1 μm.

Fig. 23. An electron micrograph of a germinating conidia, 24 h after inoculation on fourth instar larva that was fed on normal food. It shows a short germ-tube (gt) with mitochondria (m); broken conidial wall (at large arrow heads), nucleus (n), vacuole (v) and oil globules (o).
Bar = 1 μm.


22
o
l
o
pt
e

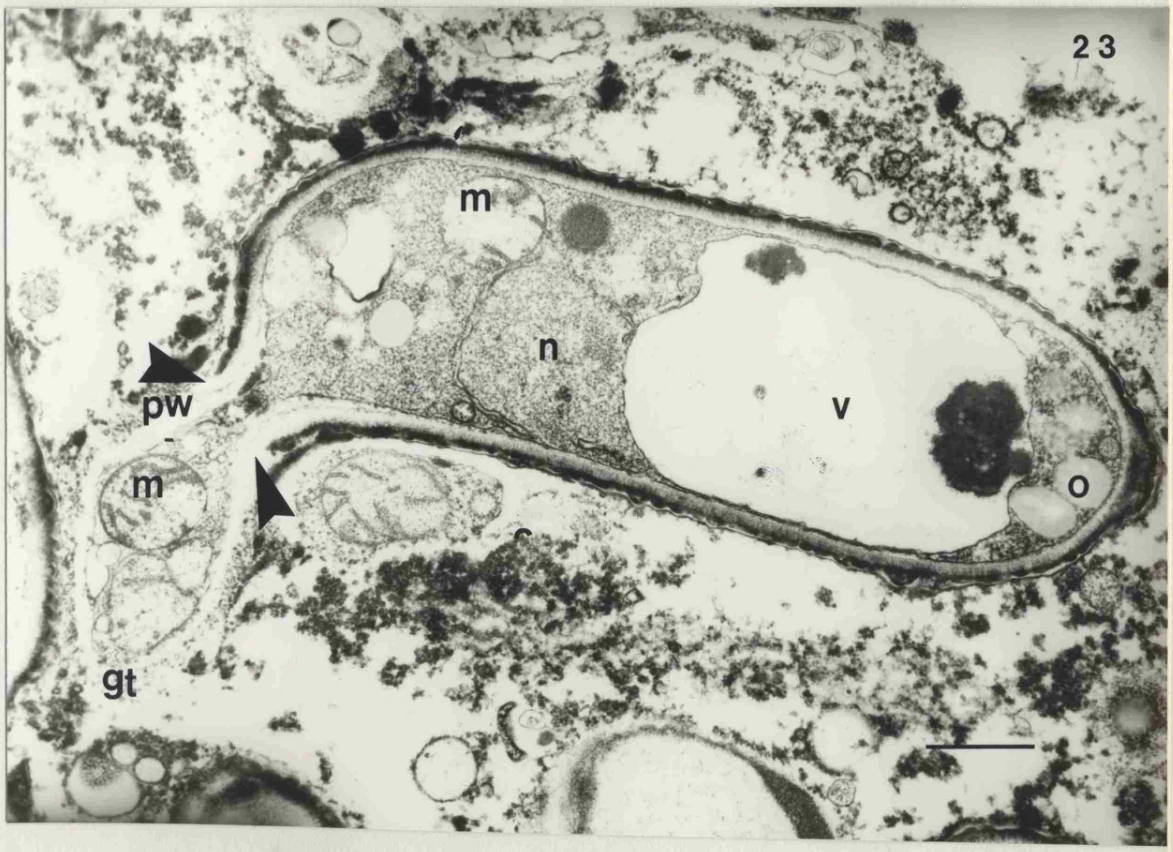
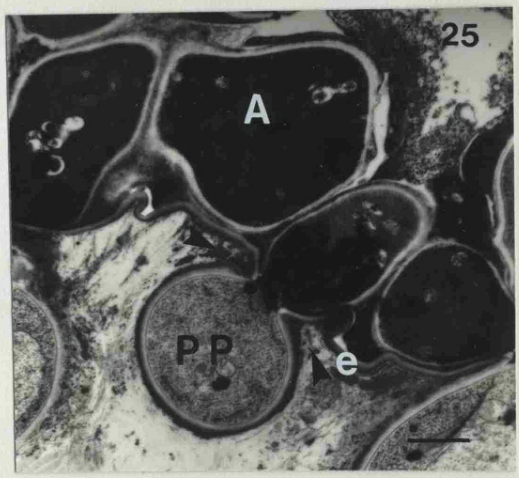

23
m
n
pw
v
o
m
gt

Fig. 24. An electron micrograph of a germ-tube forming an appressorium, 24 h after inoculation on fourth instar larva that was fed on normal food. It shows an appressorium (A) with a mitochondria (m); short germ-tube (gt), septum (s) and vacuoles (v). Bar = 1 µm. Note the electron transparent primary wall (pw).

Fig. 25. An electron micrograph of appressoria forming a penetration peg and a penetration plate, 48 h after inoculation on a fourth instar larva that was treated with Dimilin. It shows the appressoria (A), penetration peg (at arrows) and penetration plate (pp). Bar = 1 µm.
(e)=epicuticle

Fig. 26. An electron micrograph of a penetration plate, 24 h after inoculation on fourth instar larva that was treated with Dimilin. It shows insect epicuticle (e), penetration plate (pp), thin hypha (h), and histolysed cuticle (at star). Note the electron transparent primary wall (pw). Bar = 1 µm.


24
v
s
m
gt
m
A
v


25
A
P P
e

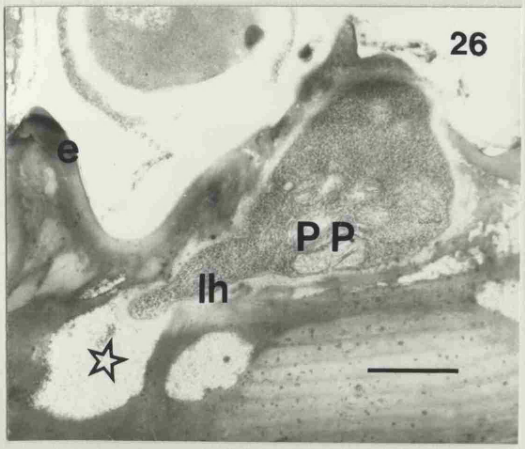

26
e
P P
lh

Fig. 27. Low power electron micrograph of the cuticle, 48 h after inoculation of fourth instar larva that was fed on ordinary food. It shows epicuticle (e), lamellated cuticle (lm), penetration hyphae (ph) and epidermis (ep). Bar = 10 µm.


27
e
Ph
Ph
lm
ep

Fig. 28. An electron micrograph showing a cross section of a penetration hypha, 48 h after inoculation of a fourth instar larvae that was fed on ordinary food. It shows the lamellated cuticle (lm), histolysed cuticle (at star), mitochondria (m) and rough endoplasmic reticulum (rer).

Bar = 1 µm.

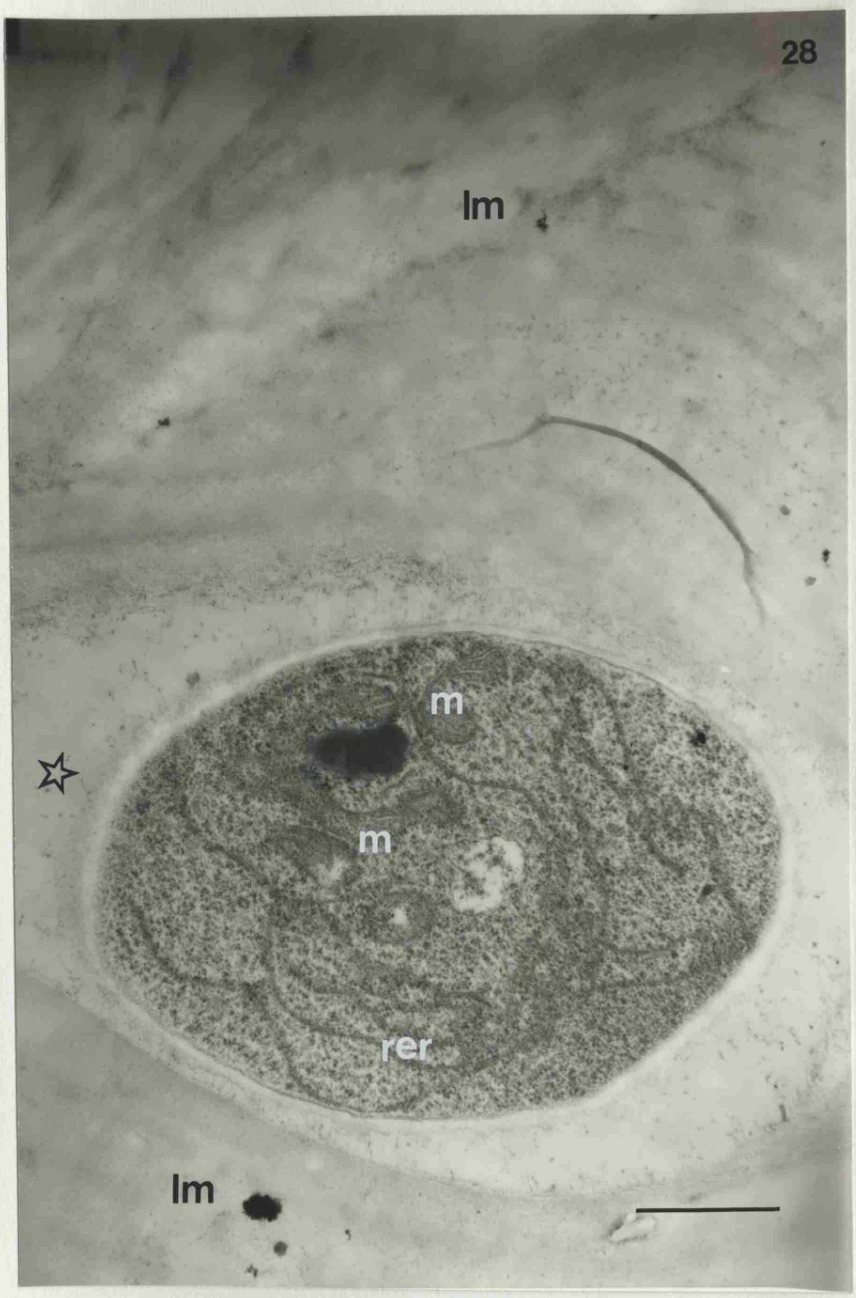

28
lm
m
m
rer
lm

Fig. 29. An electron micrograph showing a penetration hypha growing vertically through a pore canal, 48 h after inoculation of a fourth instar larva that was fed on ordinary food. It shows vacuoles (v), lipids inclusions (l), epidermis (ep), lamellated cuticle (lm) Bar = 1 µm.


29
Ph
v
lm
l
l
v

Fig. 30. An electron micrograph of a hyphal body within an epidermal cell (ep). It shows a nucleus (n), mitochondria (m), rough endoplasmic reticulum (rer), the hyphal primary wall (pw). Bar = 1 µm.

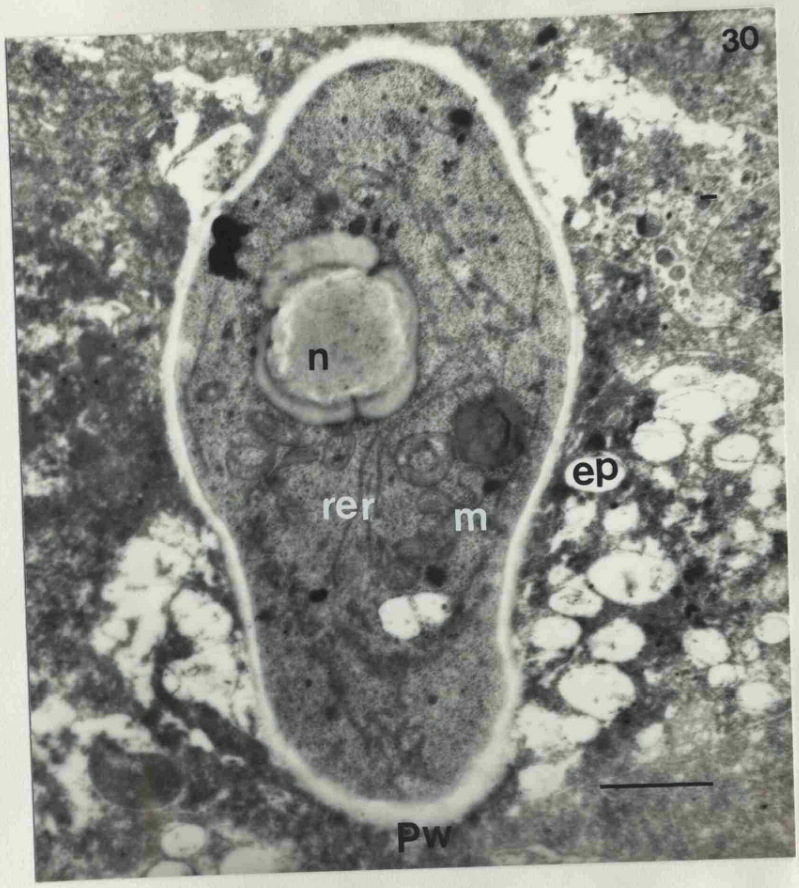
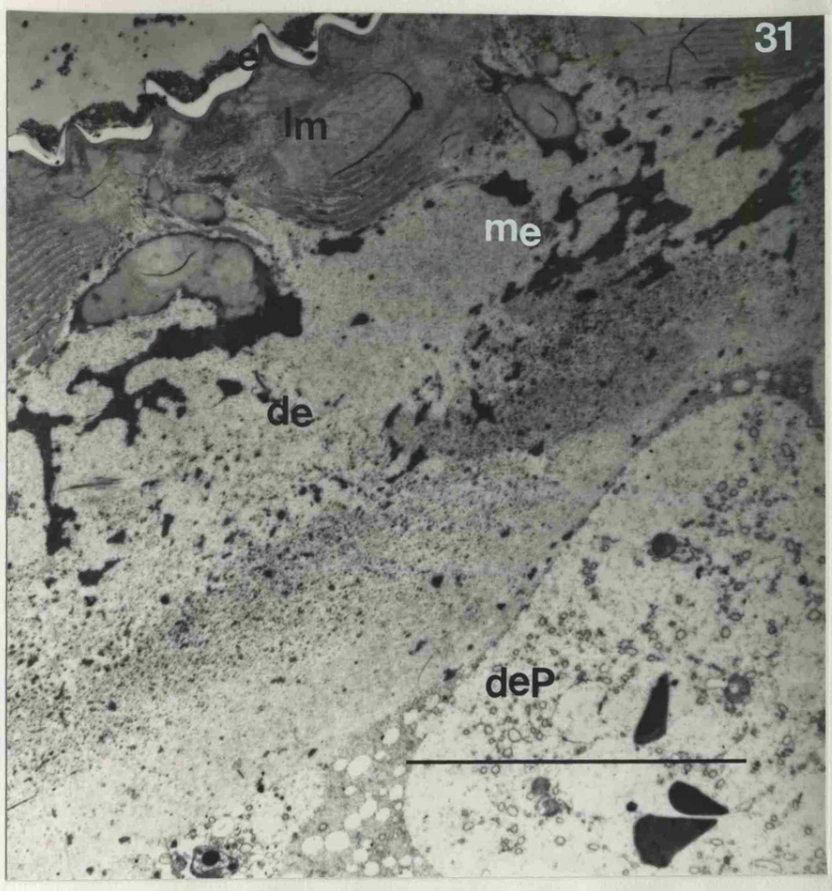

30
n
rer
m
ep
Pw

Fig. 31. Low power electron micrograph of the cuticle of a fourth larval instar, 48 h after inoculation and fed on Dimilin-treated food. It shows the epicuticle (e), lamellated cuticle (lm), disorganised cuticle (de), melanin-like bodies (me) and a disrupted epidermal cell (dep). Bar = 10 µm.

Fig. 32. An electron micrograph of a hyphal body (hb), within a disorganised region of the cuticle of a fourth instar larva, 48 h after inoculation and fed on Dimilin-treated food. It shows the nuclei (n) and melanin-like bodies (me). Bar = 10 µm.


31
lm
me
de
deP

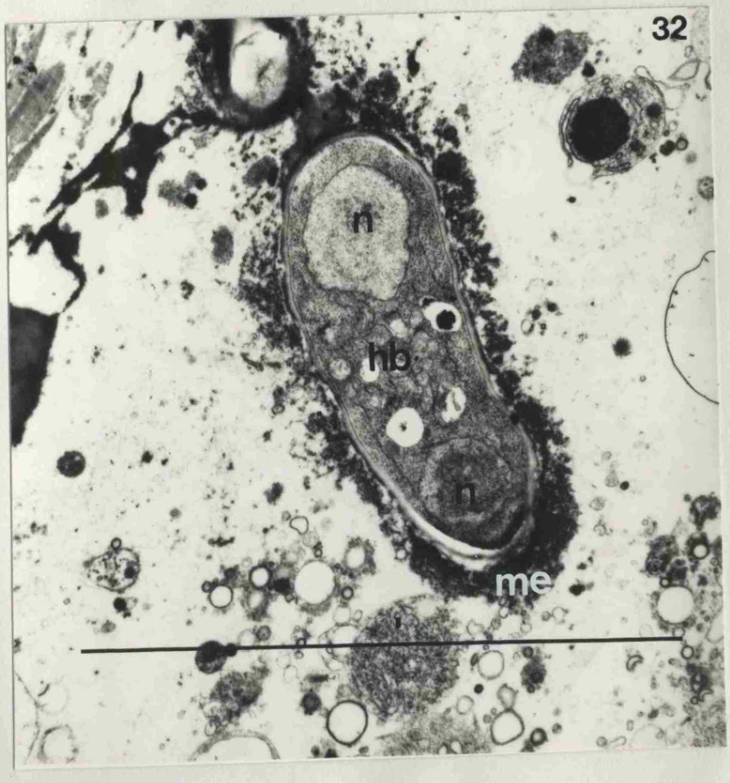
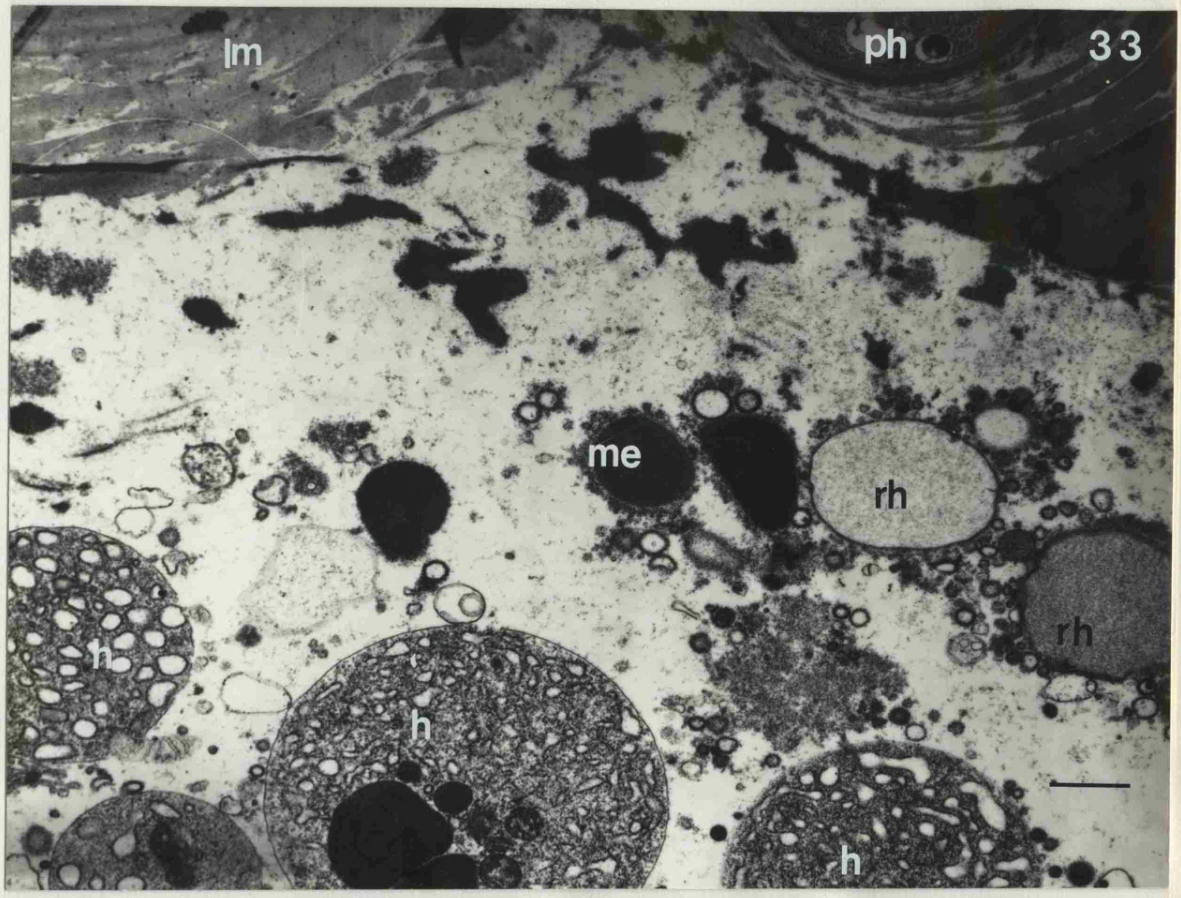

32
n
hb
n
me

Fig. 33. An electron micrograph of haemocytes within a disorganised cuticle being recruited towards a penetration hypha (ph), 48 h after inoculation, insect fed on Dimilin-treated food. It shows haemocytes (h), erupted haemocytes (rh), melanin like bodies (me) and lamellated cuticle (lm). Bar = 1 μm.

Fig. 34. An electron micrograph of haemocytes within a disorganised cuticle being recruited towards penetration hyphae (ph), 48 h after inoculation and insect fed on Dimilin-treated food. It shows aggregation of melanin-like bodies (me) and haemocytes (h). Note the disruption of lamellated cuticle (lm) and presence of melanin-like material (me) within (lm).


lm
ph
33
me
rh
rh
h
h
h

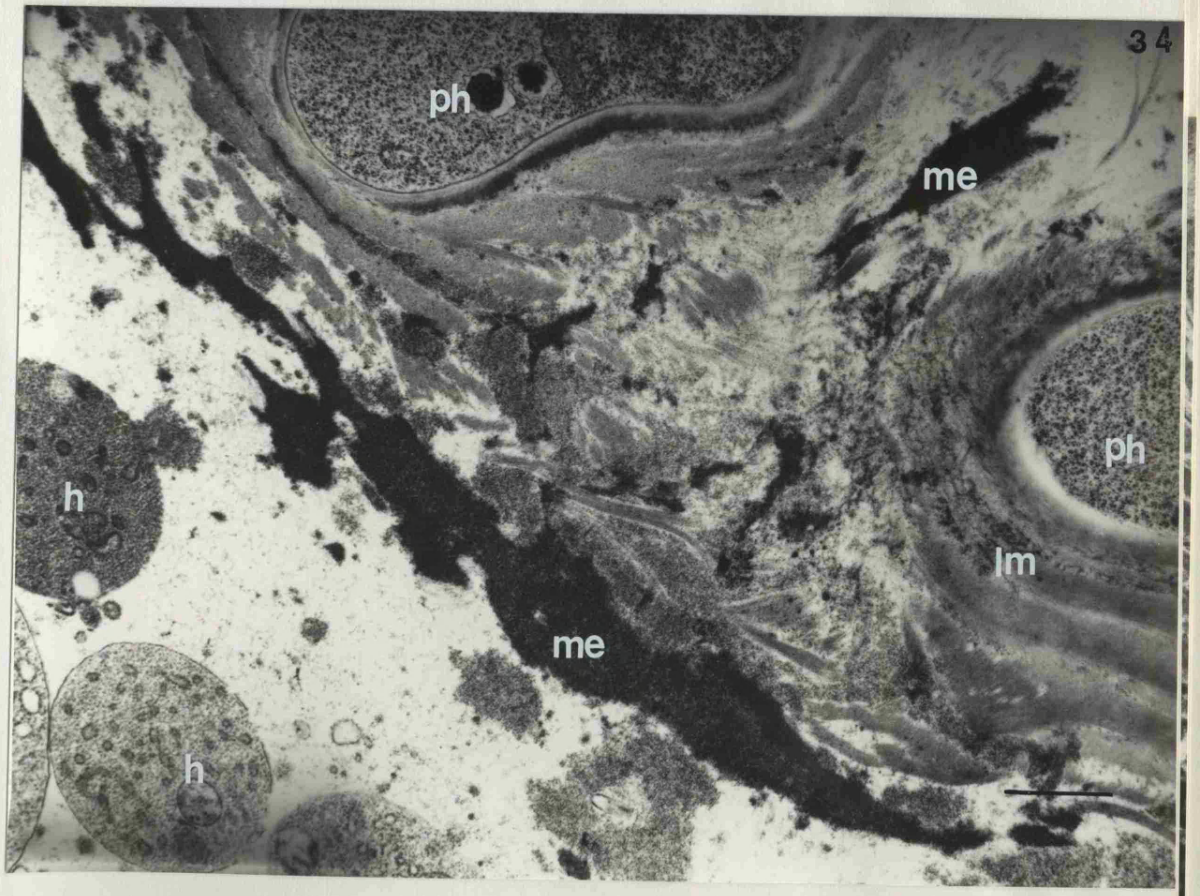
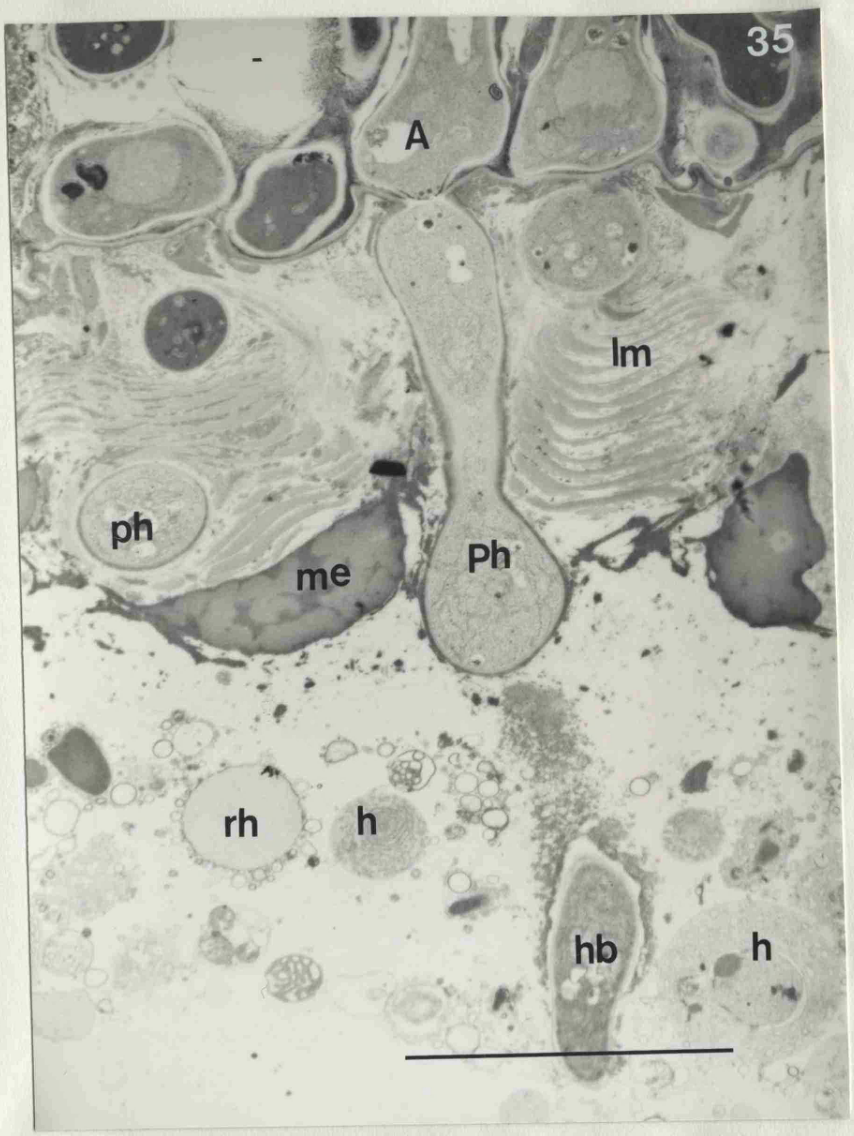

34
ph
me
ph
h
lm
me
h

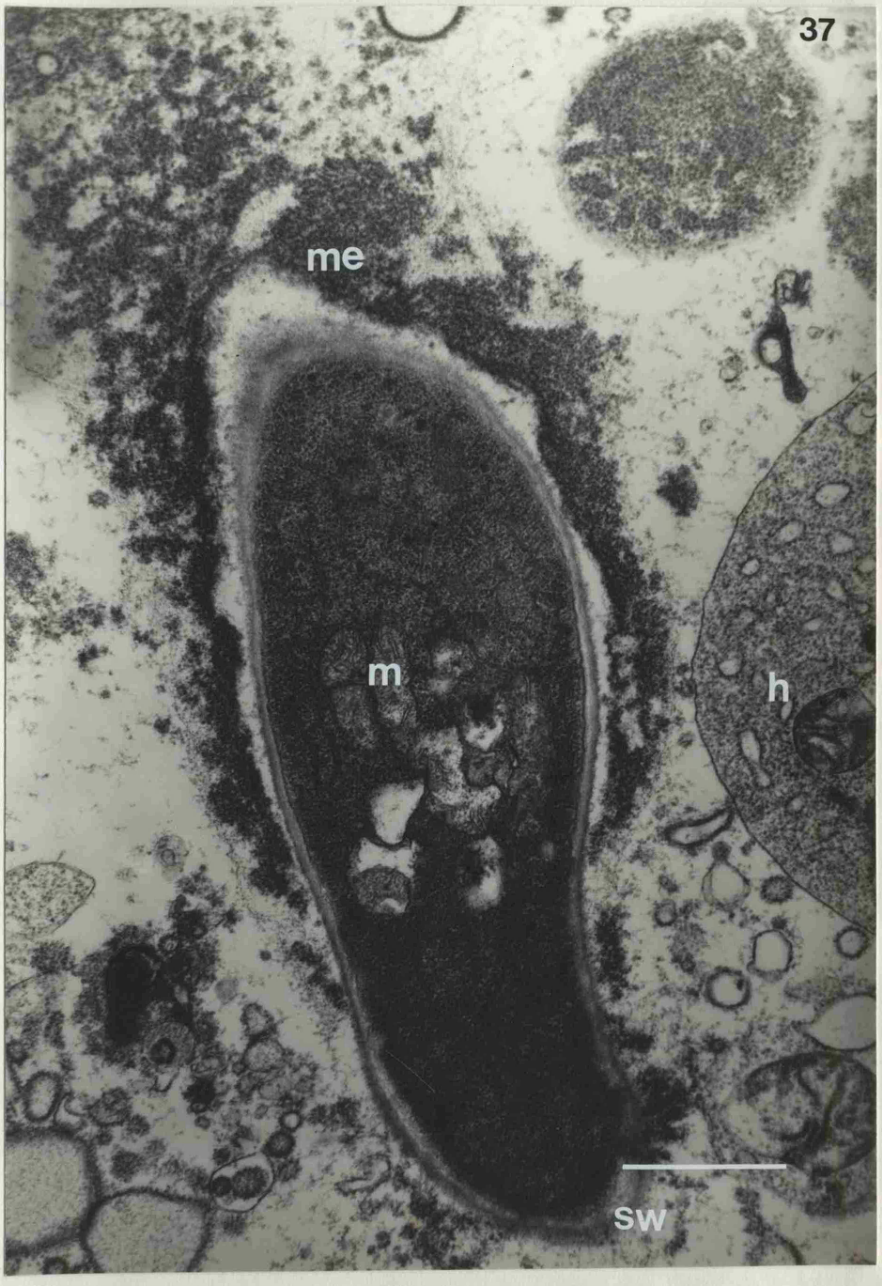
Fig. 35. Low power electron micrograph of the cuticle of a fourth instar larva, 48 h after inoculation and fed on Dimilin-treated food. It shows appressoria (A), vertical penetration through pore-canal and parallel growth of penetration hyphae (ph), budding of hyphal bodies (hb), aggregation of haemocytes (h) and lamellated cuticle (lm). Note the short penetration peg (at arrows). Bar = 10 µm.

Fig. 36. An electron micrograph highlighting the expanding terminal of the vertically growing hyphae of Fig. 35. It shows the lamellated cuticle (lm), melanin-like bodiees (me) and haemocytes (h). Note the direction of lamellation (lm) at the pore canal and compare with Fig. 9.


35
A
lm
ph
me
Ph
rh
h
hb
h


36
me
lm
Ph
h

Fig. 37. An electron micrograph showing a magnified hyphal body of Fig. 35. It shows haemocytes (h), melanin-like material (me) and mitochondria (m). Note the infiltration of melanin-like material into the hyphal body and the presence of a secondary wall (sw). Bar = 1 μm.


37
me
m
h
sw

Discussion

Ultrastructure of the cuticle of the 4th larval instar.

Cuticulin

Cuticulin in *Manduca* cuticle appears as a distinct electron-dense layer on the newly formed cuticle (Fig. 10). There are no clear lamellations probably because they are obliterated by the contents of the electron-dense filaments that traverse it (Fig. 6). Similar homogenous electron-dense cuticulin has been reported in post-ecdysial cuticle of Elaterid larvae (Zacharuk, 1972). In contrast cuticulin is often described as a trilaminar layer in Lepidopterous (Locke and Krishnan, 1971; Locke, 1975; 1976), Dipterous (Filshie, 1970; 1980) and Coleopterous insects (Zacharuk, 1972; 1976).

The dense filaments which traverse the epicuticle probably carry the precursors of a wax layer, that is secreted on the top of cuticulin. The wax layer is absent and does not stand the osmication and dehydration procedure (Locke, 1961; Filshie, 1970).

Cuticulin is formed before ecdysis (Figs. 1,2,8,9 and 10). It consists of tanned lipoprotein (Wigglesworth, 1948; 1953; Zacharuk, 1972). It functions to protect the newly formed cuticle and the epidermal cells underneath from the moulting fluid (Locke and Krishnan, 1971). Its extensive folding ensures the possibility of post-ecdysial growth (Figs 1 and 2) as suggested for the Australian blow fly, *Lucilia cuprina* (Filshie, 1970).

Protein epicuticle

The inner epicuticular layer beneath the cuticulin is known as the protein epicuticle. Its deposition is completed before ecdysis (Figs. 1, 2, 8, 9 and 10). It is a dense homogenous layer that follows the contours of the cuticulin. In *Calpodes ethlius* (Locke, 1961) and Elaterid larvae (Zacharuk, 1972) protein epiculticle is thicker at each papilla. In contrast, the protein epicuticle in *Manduca* is of uniform thickness and the papillae are filled with dense vesicles (Fig. 7).

The chemical composition and function of protein epicuticle are believed to be similar to that of the cuticulin (Locke, 1961; Zacharuk, 1972).

Procuticle

The layer between the dense epicuticle and the lamellated endocuticle has been termed the "amorphous zone" by Wolfgang and Riddiford (1981). It is transitional in nature between the dense layer of the epicuticle and the typical oriented fibrillar lamellae of procuticle (Zacharuk, 1972). In pre-exuvial cuticle it is formed as a continuation of the divergent pore-canal. The distal part of the pore-canal ramifies to fill the papillae with fibrous and filamentous material (Figs. 2 and 10), similar to that reported by Wigglesworth (1957). In post-ecdysial cuticle the filamentous

material (Fig. 10) appears to coallesce to form conspicuous dense vesicles that are distributed throughout the layer (Fig. 7). Similar vesicles in the transitory zone of *Calpodes* are thought to be made up of melanin (Locke, 1961).

Procuticle deposition starts before the moult. At the time of ecdysis some 10 - 13 lamellae are deposited with a total thickness of 8 µm (Fig. 1). By comparison, at the fifth larval ecdysis of *Manduca* the new cuticle consists of 10 lamellae, 10 - 15 µm thick (Wolfgang and Riddiford, 1981).

The cuticle increased in thickness 4-fold during the first 48 h of the fourth instar. Even greater increases in cuticular thickness have been recorded in other insects e.g. in *Calpodes* ~~*clothius*~~ ethlius the cuticle of fifth instar increases 7 - 8 times (Locke, 1965).

The increase in overall cuticular thickness is accompanied by a substantial reduction in thickness of individual lamellae. At the time of ecdysis the width of each lamella is 0.73 µm, but is reduced to 0.15 µm 24 h after ecdysis (Figs 1 and 3). In fifth larval instar of *Manduca* a 10-fold reduction in lamella thickness was found by Wolfgang and Riddiford (1981). This was due to the stretching of the cuticle during intermoult growth (Figs 4 and 5).

Subcuticle

The subcuticle is a dense granular layer at the time of ecdysis (Figs 1 and 14). The density of the layer decreases during the course of the instar (Figs 3 and 4). A similar observation was made by Zacharuk (1972) in Elaterid larvae. The fibres of the inner lamellae are seen to merge into the subcuticle layer (Fig. 17). A similar phenomenon has been seen in *Calpodes* ~~*elothius*~~ ethlius (Locke, 1961) and Elaterid larvae (Zacharuk, 1972). The subcuticle layer is thought to be the region of organisation and orientation of chitinous fibres to form the lamellated cuticle (Fig. 17). In addition Taylor and Richards (1965) suggested that the subcuticle contained a mucilaginous material binding the cuticle with the epidermis. In *M. sexta* larvae there is no ultrastructural evidence for such function.

Pore-canals

Pore canals are the most prominent feature of the pre- and post-ecdysial procuticle. In other insects the pore canals are laid down pre-ecdysially and confined to this region of the procuticle (Wigglesworth, 1957; Filshie, 1970: Zacharuk, 1976).

The pore canal consists of cytoplasmic extensions (pore-canal filaments) of the plasma membrane of the epidermis surrounded by a cuticular sheath (Fig. 9). The latter appears similar in composition to the lamellae of the procuticle. The

differences being that the fibrils are laid down perpendicular to the plane of the lamella (Figs 2 and 9).

The pore canals in the tergal cuticle of fourth instar *Manduca sexta* are similar to those described in *Tenebrio molitor* (Locke, 1961; Filshie, 1970) and *Galleria mellonella* (Locke, 1961). Therefore it is most surprising that Wolfgang and Riddiford (1981) have defined similar structure in fifth instar cuticle of *Manduca* as "cuticular columns" secreted by long microvilli , rather than pore canals. Indeed the reason for discrepancy in the interpretation of these structures between the present work and that of Wolfgang and Riddiford (1981) is not obvious.

One major problem is the different terminology employed by authors engaged in research on pore canals, e.g the cytoplasmic extensions of Wigglesworth (1948; 1953; 1957; 1976) are the same as the extended microvilli and pore-canal filaments of Locke (1961), Filshie (1970) and Zacharuk (1972). This may have contributed to the failure of Wolfgang and Riddiford (1981) to recognise the true nature of the "cuticular columns".

A cuticular sheath surrounding the pore-canal filaments (Figs. 2 and 9) has been observed in other insects such as *Tenebrio molitor, Galleria mellonella* (Locke, 1961), *Hypoderma bovis* (Kennaugh, 1965) and *Sarcophaga falculata*

(Dennell, 1943). This sheath is chitinous in nature in *Hypoderma bovis* (Kennaugh, 1965). In the latter the pore canal is large (2 - 3 µm) and chitin is deposited in a helical manner. In *Manduca* the pore-canal is wider in the outer region of the cuticle, 3 µm (Fig. 2) and narrows proximally to 1.5 µm.

Many functions have been suggested for the pore-canals. They may contribute to the formation of the epicuticle (Wigglesworth, 1948; 1953; 1957; Locke, 1961; 1975; Filshie, 1970; Zacharuk, 1972; 1976), convey digestive enzymes, chitinase and protease, through the new cuticle to digest the old (Wigglesworth, 1976), transport the moulting fluid and its digests (Wigglesworth, 1957) and assists in stretching of insect endocuticle (Wolfgang and Riddiford, 1981). In *Manduca* the pore canal ramifies as the wax canals (*sensu*; Locke, 1961) within the transitory zone (Figs. 1, 2, 9 and 10). The majority of electron-dense filaments of the wax canals merge together to form the dense vesicles (Fig. 7) while some pass through the cuticulin, presumably to form the rest of the epicuticle layers (Figs.6, and 10). Histochemical studies on larvae of *Calpodes* ~~olothius~~ ethlius have revealed phenoloxidase deposition over the papillae before ecdysis. Later phenols secreted just before ecdysis (Locke and Krishnan, 1971) fuse to form a diffuse mass of melanin in the transitory zone extending deep into the procuticle (Locke, 1961). In *Manduca* melanization appears to start three hours before ecdysis, as injections of tyrosine and DOPA into the haemocoel at this time are

rapidly incorporated into cuticular melanin (Riddiford and Hori,1981). These findings give a support to the interpretation that the dense vesicles noted in the present study are melanin.

The effect of Dimilin on the fine structure of the cuticle

General

The thickness of the cuticle from Dimilin-treated insects is similar to that of non-treated individuals (Figs. 3, 4, 19 and 20). Grosscurt (1978) found that Dimilin had no effect on the increase in thickness of elytral cuticle of *Leptinotarsa decemlineata* which normally occurred during adult life. In contrast Mitsui *et al.* (1980) found that an LD100 topical dose of Dimilin reduced the rate of post-ecdysial cuticle synthesis in fifth instar larvae. By the onset of "wandering" the endocuticle of Dimilin treated insects was only 2/3rds as thick as normal. 5 µg/ml Dimilin completely inhibited cuticle deposition *in vitro*.

In comparison to the 40 lamellae deposited within the 24 hours following ecdysis in normal insects (Fig. 3), only 3 - 4 proper lamellae are deposited in Dimilin-treated insects (Figs. 1 and 19). This indicates that only a short time is needed for the poison to take effect. *In vivo* Ker (1977) reported that the effect of Dimilin on locusts depended on the rate of feeding, the level of metabolism and the route of administration. An injected dose took 80 minutes to show

an effect. Deul *et al.* (1978) found that, *in vitro,* 15 minutes was sufficient for Dimilin to affect cuticle formation.

Procuticle

There are no lamellations in post-ecdysial cuticle of insects fed on Dimilin (Figs. 19 and 20). Similar effects of Dimilin treatment have been observed in *Pieris brassicae* (Mulder and Gijswijt, 1973; Gijswijt *et al.*, 1979); *Boarmia bistortata* (Salama *et al.*, 1976) and *Leptinotarsa decemlineata* (Grosscurt, 1978) using light microscopy.

In general the disrupted procuticle is assumed to be mainly protein (Ishaaya and Casida, 1974; Grosscurt, 1978). However, the ultrastructure of Dimilin-affected cuticle from larvae of *Manduca sexta* is open to an alternative interpretation. The majority of the cuticle in a normal insect is made up of chitin and protein (Locke, 1976). Since chitin is thought to be electron transparent, the lamellar appearance of the cuticle is attributed to the electron opacity of the protein (Neville, 1975). Therefore the electron transparent patches in Dimilin-affected cuticle could be disorganised short chain chitin fibrils. If this interpretation is correct, it adds support to the view that Dimilin does not completely inhibit chitin synthesis, but hinders its organisation (see Clarke *et al.*, 1977).

The apical (pre-ecdysial)lamellae of normal cuticle decrease in thickness during the course of an instar. This is due to plastic deformation caused by growth of the larva. There is no comparable change in the thickness of the outer layers of the cuticle in Dimilin-treated insects (Figs. 11 and 18). Presumably because growth is restricted in these animals n.b. faecal pellet production is reduced (see also Mitsui *et al.*, 1980).

Plasma membrane

The abnormalities of the epidermal cells observed in Dimilin-treated insects viz. irregular microvilli, loss of electron density of the plasma membrane plaques (Figs. 14 - 17) and the undulating topography of the plasma membrane (Fig. 19) have been reported before (Ker, 1978). However, the cytoplasmic extrusions found 48 h post-treatment do not seem to have been reported before (Figs. 15 and 20). Ishaaya and Casida (1974) explained the speed of action of Dimilin as due to a gross effect on the epidermal cells rather than a specific inhibition of chitin synthetase n.b. Dimilin has no effect on cell-free extracts of chitin synthetase (Mitsui *et al.*, 1981). Therefore, the cytoplasmic extrusions could be the result of the stimulation of the multiplication and "turn-over" of epidermal cells, analogous to the situation in actively secreting insect midguts (Abboud, 1981).

Pore-canal

The disorganisation of the cuticular structure within the pore canals supports the view that they contain chitin (Figs. 19 and 21). The abnormal pore-canal cuticle extends deep into the apical lamellae of the procuticle, which indicates that the cuticle of the distal end of the pore-canal is laid down during treatment with Dimilin i.e. post ecdysially Thus cuticle deposition in the pore-canal is not synchronised with the synthesis of the lamellae of the procuticle. A conclusion also reached by Wolfgang and Riddiford (1981), who followed the incorporation of H^3 glucose and H^3 leucine using autoradiography.

The disorganisation of the pore-canal cuticle"unmasks" the filaments and results in the plugging of pore-canals by clots of melanin (Fig. 21). The cytoplasmic components of the pore-canals (the long-microvilli) are unaffected by the insecticide and are found extending into the disorganised Dimilin-affected cuticle (Figs. 8, 9 and 19).

Ecdysial membrane

Ecdysial membrane is a terminology that was first suggested by Passoneau and Williams (1953) for a resistant layer that lined the exuvia. It is chitinous in nature (Passoneau and Williams, 1953), formed from the inner lamellae of the procuticle, and is remarkably resistant to moulting fluid

enzymes (Malek, 1958). Taylor and Richards (1965) described it as a discrete layer that was secreted beneath the old endocuticle. It is not a consistent feature of moulting insects (Malek, 1958), being absent from Dipteran larvae, where little digestion of the endocuticle takes place (Zacharuk, 1976). The function of the ecdysial membrane is unknown but it may retain the delamellated residue of the hydrolysed endocuticle prior to its loss with the exuvia (Zacharuk, 1976). Alternatively, Locke and Krishnan (1971) suggested that ecdysial membrane formation was an inevitable stage in the moulting sequence and there was no specific function for it. In *Manduca* the ecdysial membrane of Dimilin treated insects is loose and thick, but never-the-less appears as a discrete layer (Figs 12 and 13). Perhaps Dimilin could be used to further investigate the nature, origin and function of the ecdysial membrane.

The fine structure of *Metarhizium anisopliae* and its penetration of the cuticle of the fourth larval instar

The penetration of insect cuticle by entomopathogenic fungi is generally considered to have enzymic and mechanical components (Ferron, 1978; Roberts and Humber, 1981). The present fine structural study provides evidence that is consistent with such a view. The apparent constriction of the penetration peg during passage through the epicuticle (Figs. 25 and 35) and the swelling of the tip in the procuticle

(Fig. 25) has been interpreted in other *Metarhizium*-insect combinations as the results of the hypha being under pressure during mechanical penetration of the outer region of the cuticle, followed by expansion in the cavity created within the softer inner layers by enzymic action (Robinson, 1966). However, the lack of distortion of the epicuticle during initial penetration (Fig. 25) suggests that hydrolysis may also take place. Displacement of procuticular lamellae by lateral penetrant hyphae (Fig. 28) is also a clear indication of mechanical penetration (McCauley *et al.*, 1968; Lambiase and Yendol, 1977; Mohammed *et al.*, 1978). But here too, clearing zones around the hyphae (Figs. 27 and 28) are suggestive of enzymic degradation of the cuticle (Robinson, 1966; Zacharuk, 1970; 1973; Boucias and Pendland, 1982).

Zacharuk (1970; 1973) found that growth of *M. anisopliae* through the cuticle of Elaterids followed a step wise progression. Lateral growth between lamellae, in the form of one or more layers of penetrant hyphal bodies, alternated with short, thin vertical hyphae which crossed the cuticular lamellae. By contrast lateral growth of *Metarhizium* in *Manduca sexta* cuticle consisted entirely of individual penetrant hyphae (Fig. 27) which apparently did not give rise to distinct vertical penetrant hyphae. Presumably penetration was achieved by oblique growth of lateral penetrant hyphae across cuticular lamellae. The different mode of penetration of *Metarhizium* within the two species of insects

would reflect a difference in cuticular form and chemistry. Elaterid (Coleopteran) cuticle is considerably thicker and harder (more sclerotised) than *Manduca* (Lepidopteran) cuticle. As a consequence not only will Elaterid cuticle provide fewer (available) nutrients (cross-linking of proteins during sclerotization confers resistance to hydrolysis (Lipke and Geoghegon, 1971) but it will present a greater physical barrier to penetration. In the light of this the short, lateral hyphal bodies and the vertical penetrant hyphae of *Metarhizium* within Elaterid cuticle may be adaptations to provide maximum absorption of available nutrients (increased surface area) and most efficient means of producing mechanical pressure, both being unnecessary within the more favourable environment of *Manduca* cuticle.

To date there has been little evidence for fungal penetration of insect cuticle via natural channels apart from spiracles and sense organs (David, 1967). In particular pore canals in many insects are too small to be a vehicle for fungal penetration, though in Elaterids pressure points seem to develop over the ends of the wax canal filaments during initial formation of the penetration peg (Zacharuk, 1973). However, in *Manduca sexta* pore canals and penetrant hyphae are of comparable size and when a hypha grows into a pore-canal vertical penetration ensues (Figs. 29 and 35). Pore canals in *Manduca* consist of fine filaments embedded in a matrix of cuticular fibrils (see above). Thus they do not provide a simple channel for fungal growth, but must

generate less mechanical resistance to vertical progress than the rest of the cuticle, because the fibrils are laid down, at right angles to the plane of the cuticle (Figs. 2, 3 and 9). The relative ease of vertical penetration through the pore-canal may also help account for the failure to observe production of vertical hyphae from lateral hyphae in the rest of the cuticle (see above).

Pore canals in the cuticle of *Manduca* are normally capped with a diffuse mass of melanin (Fig. 7). The last named is known to have antimicrobial properties (Gotz and Vey, 1974; Unestam and Nyhlen, 1980; Unestam and Weiss, 1970; Kuo and Alexander, 1967), and there is evidence that cuticular melanin may interfer with fungal invasion in crayfish (Unestam and Weiss, 1970; Unestam and Nyhlen, 1980; Ajaxon and Soderhall, 1982). However, in the present case the presence of melanin at the top of the pore-canals clearly does not prevent *M. anisopliae* invading *Manduca* via this route.

Fine structure of the penetration of Dimilin-affected cuticle of fourth instar larvae by *Metarhizium anisopliae*

The cuticle of Dimilin-treated insects affords little resistance to penetrant hyphae of *Metarhizium* (Fig. 35). The reasons for this appears to be as follows. The post-ecdysial cuticle is disorganised by the action of the Dimilin and lacks strength (see earlier). It was established above that

the pore canals are sites for invasion by *Metarhizium*. The cuticular sheath of the pore canal filaments in *Manduca* appears to be laid down post-ecdysially because the proximal region of the pore-canal within the pre-ecdysial cuticle is disrupted by treatment with Dimilin (see above and Fig. 21). This will provide a weakened direct route through to the epidermis.

The thickness of the cuticle in the dual treatment is considerably reduced (19 µm) with respect to that in normal insects (34 µm), but similar to that in insects treated only with conidia (24 µm) (see Figs. 4, 20, 27 and 31). It was noted earlier that reduced cuticular thickness in *Leptinotarsa decemlineata* infected with *Beauveria bassiana* was attributable to a toxic action of the fungus on the epidermis (Vey and Fargues, 1977). Whatever the reason for the similar phenomenon observed in the present work, and note that the epidermal cells are disrupted in inoculated insects (Fig. 31), it must facilitate the penetration of *Manduca* cuticle by *Metarhizium*.

Post-ecdysial Dimilin-treated cuticle is almost completely destroyed, presumably by the action of hydrolytic enzymes. It is not immediately obvious why the Dimilin-treated cuticle should be so susceptible to digestion. In normal cuticle, clearing zones around penetrant hyphae are restricted in extent (Figs. 27, and 28). The extracellular enzymes are released by penetrant hyphae. The area of degradation could

normally be limited by molecular sieving (large enzymes and small intermolecular spaces) (Charnley, 1983). In which case, the disorganisation of the Dimilin-affected cuticle might allow free movement of the hydrolytic enzymes and wholesale degradation of the cuticle.

Haemocytes appear within the remains of the Dimilin-affected cuticle (Figs. 32 - 35). Aggregation of blood cells around penetrant hyphae of parasitic fungi in arthropods has been observed many times (Glaser, 1926; McCauley *et al.*, 1968; Unestam and Nylund, 1972). The recruitment of haemocytes in the present case may be in response to the disruption of the epidermis (Fig. 31). Recruited haemocytes are mainly granulocytes and spherulocytes (Jones, 1958). These types of cell often increase in number when a pathogen is inoculated into an insect haemocoel (Peter *et al.*, 1978).

Granular haemocytes congregated in the vicinity of the penetrant hyphae and hyphal bodies (Figs. 32 - 35). Flocculent material and dense globules are also present, possibly derived from the granular haemocytes (Figs. 33 and 34). Extensive dense melanin-like material may represent at least in part melanization of the flocculent material (Figs. 33 and 34) (Boman, 1981), brought about by the release of appropriate enzymes (Tyrosinase; Jones (1956); phenoloxidase; Unestam and Nylund (1972); Nappi (1973)) and substrates (Tyrosine and DOPA, (Salt, 1970; Nappi, 1973)) from the granular blood

cells. However, melanin-like material is present throughout the cuticle of the dual treatment, both in regions where penetrant hyphae and haemocytes are present (Figs. 32, 33, 34, 35 and 37) and those where they are not (Fig. 31). It was suggested above that larger intermolecular spaces in the Dimilin-treated cuticle could allow greater movement of fungal enzymes. This could include fungal proteases responsible for the widespread activation of host pro-phenoloxidase and subsequent melanization within the cuticle (Pye, 1974).

Whatever the origin of the melanin-like material it appears to be attracted to the surface of the penetrant hyphae and hyphal bodies (Figs. 32, 33, 34, and 37). However, invasion of the disrupted cuticle by haemocytes and associated events occurs too late to influence the fungus which proceeds to invade and kill the insect.

APPENDIX 1

Ingredients of artificial diet

Pre-mix components	Weight in g
wheat germ	750
casein	350
sucrose	300
dry yeast	150
Wesson's salt mixture	100
sorbic acid	015
cholesterol	010
methyl-p-hydroxybenzoate	010
choline chloride	010

These were usually kept in an airtight plastic container at room temperature.

For a batch of 504 g of a pre-mix other ingredients were added as follows:

Other ingredients	Weight in g or Volume in ml
ascorbic acid	12
aureomycin	0.30
Vanderzant's vitamins mixture	0.30
10% formaldehyde	12
raw linseed oil	6
vegetable oil	6
agar	45

Diet preparation

A batch of 504 g of pre-mix was put in a Waring blender, a 1250 ml of boiling distilled water was added and mixed for ca. 5 minutes, meanwhile the oils and 10% formaldehyde was added. Agar was cooked separately in a 1250 ml of distilled water and transferred while it was hot to the Waring blender and mixed further with the pre-mix.

When the temperature cooled to 70°C, or below, the ascorbic acid, aureomycin and Vanderzant's vitamins mixture were added. The diet was then poured into containers lined with aluminium foil and allowed to cool and solidify at room temperature before being stored at 4°C.

When required, the diet was moved out of the fridge and put at room temperature for 2 - 3 hours before being introduced to insects.

APPENDIX 2

Table 1A. Effect of Dimilin on first instar larvae of *Manduca sexta*, insecticide applied as a stomach poison to artificial diet, insects maintained at low relative humidity (RH)

Concentration of Dimilin % ai (w/v)	Number of insects used	% mortality
0.031	24	100
0.015	54	97
0.007	78	72
0.0035	78	46
0.0018	54	17
0.0	78	4

Table 2A. Effect of Dimilin on second instar larvae of *Manduca sexta* when applied as stomach poison to artificial diet - insects maintained at low RH

Concentration of Dimilin % ai (w/v)	Number of used	% mortality	% failure to ecdyse
0.02	20	100	100
0.01	20	90	90
0.005	20	85	70
0.0025	20	45	45
0.0	20	0	0

Table 3A. Effect of Dimilin on third instar larvae of *Manduca sexta* when applied as stomach poison to artificial diet - insects maintained at low RH

Concentration of Dimilin % ai (w/v)	Number of insects used	% mortality	% failure to ecdyse
0.062	22	100	100
0.031	22	96	96
0.015	22	86	81
0.007	21	76	67
0.0035	22	59	54
0.0	22	5	5

Table 4A Effect of Dimilin on fourth instar larvae of *Manduca sexta* when applied as stomach poison to artificial diet - insects maintained at low RH

Concentration of Dimilin % ai (w/v)	Number of insects used	% mortality	% failure to ecdyse
0.062	12	100	100
0.031	13	100	100
0.015	15	80	80
0.007	14	69	54
0.0035	15	63	50
0.0	13	0	0

Table 5A Effect of Dimilin on first instar larvae of *Manduca sexta* when applied as stomach poison to artificial diet - insects maintained at high RH

Concentration of Dimilin % (w/v)	Number of insects used	% mortality	% failure to ecdyse
0.031	20	100	65
0.015	20	90	45
0.007	20	80	40
0.0035	20	50	5
0.0018	20	10	5
0.0	20	5	5

Table 6A. Effect of Dimilin on second instar larvae of *Manduca sexta* when applied as stomach poison to artificial diet - insects maintained at high RH

Concentration of Dimilin % ai (w/v)	Number of insects used	% mortality	% failure to ecdyse	% pre-moult death
0.031	80	100	100	88
0.015	80	99	95	77
0.007	80	96	89	45
0.0035	80	90	75	15
0.0018	80	52	29	3
0.0	80	10	5	2

Table 7A. Effect of Dimilin on second instar larvae of *Manduca sexta* when applied as stomach poison to artificial diet - insects maintained at high RH

Concentration of Dimilin % ai (w/v)	Number of insects used	% mortality	% failure to ecdyse	% pre-moult death
0.01	65	100	100	65
0.005	65	96	85	46
0.0025	65	85	56	10
0.00125	65	61	36	3
0.000625	65	41	26	8
0.0	65	10	5	4

Table 8A. Effect of Dimilin on second instar larvae of *Manduca sexta* when applied as stomach poison to tomato leaves - insects maintained at high RH

Concentration of Dimilin % ai (w/v)	Number of insects used	% mortality	% pre-moult death
0.031	80	90	84
0.015	80	91	78
0.007	80	88	65
0.0035	80	80	41
0.0018	80	68	23
0.0	80	3	3

Table 9A. Effect of Dimilin on second instar larvae of *Manduca sexta* when applied as stomach poison to tomato leaves - insects maintained at high RH

Concentration of Dimilin % ai (w/v)	Number of insects used	% mortality	% failure to ecdyse
0.01	60	98	92
0.005	60	98	80
0.0025	60	78	63
0.00125	60	67	37
0.000625	60	60	47
0.0	60	5	0

Table 10A. Effect of Dimilin on second instar larvae of *Manduca sexta* when sprayed as stomach poison to tomato leaves - insects maintained at low RH - glasshouse

Concentration of Dimilin % ai (w/v)	Number of insects used	% mortality
0.02	30	100
0.01	30	100
0.005	30	100
0.0025	30	83
0.00125	30	63
0.0	30	3

Table 11A Effect of Dimilin on second instar larvae of *Manduca sexta* when sprayed as stomach poison to tomato plants - insects maintained at low RH - glasshouse

Concentration of Dimilin % ai (w/v)	Number of insects used	% mortality
0.01	80	98
0.005	80	93
0.0025	80	78
0.00125	80	44
0.000625	80	21
0.0	80	3

Table 12A Effect of Dimilin on second instar larvae of *Manduca sexta* when sprayed as stomach poison on tomato plants - insects maintained at low RH - Environmental cabinet

Concentration of Dimilin % ai (w/v)	Number of insects used	%mortality
0.01	40	100
0.005	40	98
0.0025	40	82
0.00125	40	44
0.000625	40	32
0.0	40	8

Table 13A. Effect of Dimilin on first instar larvae (6h ± 6 h) of *Manduca sexta* when applied as contact poison - insects maintained on artificial diet at low RH

Concentration of Dimilin % ai(w/v)	Number of insects used	% mortality	% failure to ecdyse
0.031	48	96	32
0.015	61	51	34
0.007	53	35	21
0.0035	53	14	5
0.0018	54	9	5
0.0	61	2	2

Table 14A. Effect of Dimilin on first instar larvae (6 h ± 6 h) of *Manduca sexta* when applied as contact poison - insects maintained on artificial diet at low RH

Concentration of Dimilin % ai (w/v)	Number of insects used	% mortality	% failure to ecdyse
0.025	24	83	62
0.019	20	80	10
0.014	20	50	5
0.011	20	45	15
0.008	22	32	26
0.0	20	0	0

Table 15A. Effect of Dimilin on first instar larvae (42 h ± 6 h) of *Manduca sexta* when applied as contact poison - insects maintained on artificial diet at low RH

Concentration of Dimilin % ai (w/v)	Number of insects used	% mortality	% failure to ecdyse
0.031	24	92	68
0.015	24	83	8
0.007	24	75	17
0.0035	24	75	12
0.0018	24	13	0
0.0	24	0	0

Table 16A. Effect of Dimilin on second instar larvae (6 h ± 6 h) of *Manduca sexta* when applied as contact poison - insects maintained on artificial diet at low RH

Concentration of Dimilin % ai (w/v)	Number of insects used	% mortality	% failure to ecdyse
0.04	30	87	47
0.02	30	63	17
0.01	55	46	13
0.005	55	24	11
0.0025	24	17	13
0.0	54	4	2

Table 17A. Effect of Dimilin on second instar larvae (42 h ± 6 h) of *Manduca sexta* when applied as contact poison - insects maintained on artificial diet at low RH

Concentration of Dimilin % ai (w/v)	Number of insects used	% mortality	% failure to ecdyse
0.062	26	100	92
0.031	55	91	53
0.015	54	65	26
0.007	52	48	10
0.0035	53	38	9
0.0	52	2	0

Table 18A. Effect of Dimilin on third instar larvae of *Manduca sexta* when applied as contact poison - insects maintained on artificial diet at low RH

Concentration of Dimilin % ai (w/v)	Number of insects used	% mortality	% failure to ecdyse
0.062	20	100	35
0.031	20	75	20
0.015	20	60	15
0.007	20	20	5
0.0035	20	10	0
0.0	20	0	0

Table 19A. Effect of Dimilin on fourth instar larvae (6 h ± 6 h) of *Manduca sexta* applied as contact poison - insects maintained on artificial diet at low RH

Concentration of Dimilin % ai (w/v)	Number of insects used	% mortality	% failure to ecdyse
0.62	14	100	100
0.31	14	79	64
0.062	14	71	71
0.031	14	36	29
0.015	14	14	7
0.0	14	7	7

Table 20A . Effect of Dimilin on first instar larvae of *Manduca sexta* when applied as contact poison - insects maintained on artificial diet at high RH

Concentration of Dimilin % ai (w/v)	Number of insects used	% mortality	% failure to ecdyse
0.025	20	65	10
0.019	20	65	20
0.014	20	55	10
0.011	20	20	5
0.008	20	10	0
0.0	20	0	0

Table 21A. Effect of Dimilin on second instar larvae of *Manduca sexta* when applied as contact poison - insects maintained on artificial diet at high RH

Concentration of Dimilin % ai (w/v)	Number of insects used	% mortality	% failure to ecdyse
0.04	55	100	85
0.02	55	98	58
0.01	55	78	32
0.005	55	37	9
0.0025	55	18	2
0.0	55	9	9

APPENDIX 3

Effect of dipping second instar larvae of *Manduca sexta* in conidial suspensions of *Metarhizium anisopliae* - insects maintained on tomato leaves at high relative humidity

Table 1A Assay 1

Concentration of conidial suspension ($x10^5$/ml)	Number of insects used	% Mortality	% Mycosis	% pre-moult death
80	60	98	95	95
40	60	100	100	100
20	60	87	87	87
10	60	97	97	93
5	60	77	75	68
0	60	0	0	0

Table 2A Assay 2

Concentration of conidial suspension x 10^5/ml	Number of insects used	% Mortality	% Mycosis	% pre-moult death
40	30	100	90	100
20	75	89	80	83
10	75	92	81	88
5	75	88	75	80
2.5	45	71	69	53
1.25	45	56	56	47
0.0	75	0	0	0

Table 3A Assay 3

Concentration of conidial suspension $x10^5$/ml	Number of insects used	% mortality	% mycosis	% pre-moult death
62.5	40	100	100	100
15.625	40	98	93	88
3.80625	40	88	80	68
0.97756	40	45	20	20
0.24414	40	8	3	0
0.0	40	0	0	0

Table 4A Assay 4

40	40	100	100	100
10	40	95	95	75
2.5	40	58	55	38
0.625	40	30	25	10
0.15625	40	20	13	10
0.0	40	0	0	0

Table 5A Assay 5

4	25	80	64	56
1	25	48	44	32
0.25	25	24	12	12
0.0625	25	12	0	0
0.0	25	7	0	0

Effect of an intrahaemocoelic injection of conidia on third instar larvae of *Manduca sexta*

Number of conidia injected/larva x 10^3	Number of insects used	% mortality
Table 6A Assay 1.		
400	10	100
40	10	100
4	10	80
0.4	10	70
0.04	10	40
0.004	10	10
0.0	10	0
Table 7A. Assay 2		
32.5	20	90
3.25	20	85
0.325	20	65
0.0325	20	60
0.0	20	0

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